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DELAYED RESORPTION OF VARIOUS FORMS OF INSULIN[1]

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FISHER and Scott (1) showed that there was little if any difference in the duration of hypoglycemia produced by intramuscular injection of an isoelectric suspension of regular insulin as compared with its acid solution, while a suspension of insulin crystals had "a slightly delayed action." Hallas-Møller *et al.* (2) say the latter effect is not significantly different. The action of zinc in producing a delayed action of insulin has been the subject of intense investigation. The delay in administering acid solutions is described by Fisher and Scott (1) and Fazekas and Himwich (3) and in administering solutions at the pH of the body fluids by Bischoff and Maxwell (4) and Hallas-Møller (5). According to Hallas-Møller (2), 1.3% zinc would be the minimum amount of zinc required to delay resorption of the insulin used.

It was rather unexpected that the precipitate (insulin OU (6)) formed by adding water to a freshly prepared urea solution of insulin has a delayed physiologic action, because such suspensions may appear amorphous even though the original insulin was crystalline and the zinc content is not greater than that found in regular crystalline insulin.

The present report is a comparative study of the biologic response of suspensions of amorphous, regular crystalline, urea insulin OU, and insulin Lente Iletin given by the intramuscular route in rabbits.

METHODS AND RESULTS

Zinc insulin crystals, supplied by Armour and Company and Eli Lilly and Company, assayed respectively 24.3 and 27 units per mg. The amorphous insulin was prepared by precipitating a crystalline insulin at the isoelectric point, washing the precipitate with ethanol and air drying the washed precipitate to a glassy mass. The bioassays were per-

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formed by cross matching in 10 to 12 rabbits. These procedures with the statistical treatment were previously described (7). The micro sugar determination was a combination of the Nelson-Somogyi procedures (8). The concentration of the insulin as injected intramuscularly was U 40 in all cases.

Experiment 1, Table 1, confirms Scott showing that an amorphous insulin when injected at the isoelectric point produces no delay in action. Experiment 2 was a repetition of Experiment 1, using a suspension of insulin crystals of uniform size instead of amorphous insulin. The results of the delayed action are highly significant and more dramatic than those of Scott

TABLE 1. INFLUENCE OF VARIOUS FORMS OF INSULIN ON THE PROLONGATION OF THE BLOOD SUGAR RESPONSE IN RABBITS ON INTRAMUSCULAR INJECTION

Exp.	Type of insulin administered	Blood sugar in mg./100 ml.		
		3 hr.	6 hr.	9 hr.
1	Amorphous insulin, pH 3, 1.5% glycerol, soluble	30	64	93
	Same adjusted to pH 5.6, with $NaHCO_3$, insoluble	30	70	92
		0 ± 1.4	−6 ± 9	1 ± 3
2	Crystalline insulin, pH 2, 1.5% glycerol, soluble	31	79	92
	Same adjusted to pH 6.5 with $NaHCO_3$, crystalline, insoluble	25	35	76
		6 ± 3.5	44 ± 7.3	16 ± 8
3	0 hr. 40% urea treated amorphous insulin, 1.5% glycerol, pH 3.0, soluble	29	71	90
	0 hr. 40% urea treated amorphous insulin, 1.5% glycerol, pH 6.6, insoluble	26	37	84
		3 ± 2.3	34 ± 9.5	6 ± 3.3
4	0 hr. 40% urea treated amorphous insulin + Zn,* 1.5% glycerol, pH 3.0, soluble	21	55	82
	0 hr. 40% urea treated amorphous insulin + Zn, 1.5% glycerol, pH 6.4, insoluble	26	25	68
		−5 ± 1.4	30 ± 7.6	14 ± 5.5
5	Regular crystalline insulin, pH 3.0, soluble	31	92	91
	0 hr. 40% urea treated crystalline insulin, 0.9% NaCl, pH 3.0, soluble	31	84	95
		0 ± 2	8 ± 7	−4 ± 3
6	Regular crystalline insulin, pH 3.0, soluble	38	95	96
	0 hr. 40% urea treated crystalline insulin, 0.9% NaCl, pH 6.3, insoluble	30	41	77
		8 ± 2.2	54 ± 4	19 ± 8
7	Lente Iletin	38	42	58
	0 hr. 40% urea treated crystalline insulin, 1.5% glycerol, pH 6.2	34	54	85
		4 ± 4	−12 ± 6	−27 ± 7
8	NPH insulin	48	58†	85

* 2 mg. per 1000 units.
† 5 hr.

and do not bear out the contention of Hallas-Møller that the delay is not significant. In Experiment 3 an amorphous insulin was dissolved in 40% aqueous urea solution at room temperature and immediately precipitated by addition of water. A suspension of this washed insoluble precipitate was compared with an acid solution of the same insulin. A marked prolongation in hypoglycemia is noted. Experiment 3 was repeated, adding 2 mg. of Zn acetate to the urea solution. It is well established as previously cited that Zn will prolong the action of insulin by forming an insoluble combination. This is indicated in the response of the acid solution of Experiment 4. However, the further delay produced by the precipitate is no greater than that produced without zinc. Experiments 5 and 6 are concerned with the action

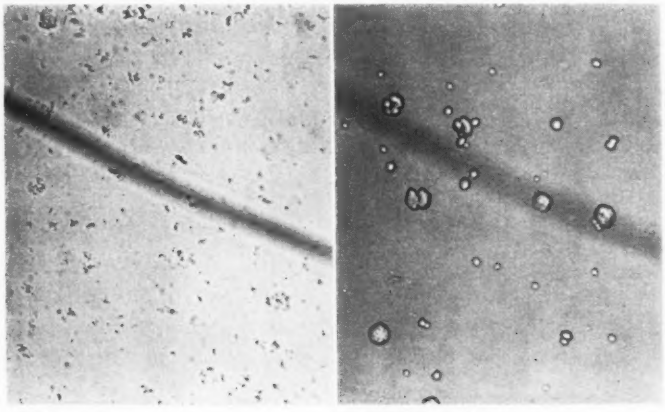

FIG. 1. OU insulin left, and regular insulin crystals right. Both these suspensions produced the same delay in hypoglycemic activity as compared with regular insulin by intramuscular injection in rabbits. ×400.

of insulin OU (made from crystalline insulin). The acid solution of this precipitate has no delay in action when compared with regular insulin, while the precipitate has a marked delay in action. In Experiment 7 Lente Iletin is compared with a suspension of OU insulin. Under magnification the crystals in the Lente Iletin preparation appeared ten times the size of the particles of the OU insulin. In this case the OU insulin appeared semi-crystalline. The Lente Iletin insulin has the more prolonged effect. Experiment 8 are data previously published (7).

The conclusion to be reached from this study is that suspensions of regular crystalline insulin or of non-crystalline OU (urea-treated) insulin may show a highly significant delayed resorption rate when given intramuscularly to rabbits. While showing less delay than that produced by Lente Iletin they approximate the curve for NPH insulin. The delay produced by insulin OU is apparently neither dependent upon size of particle nor crystalline form (see Fig. 1). Either this insulin has suffered some sort of change

in space arrangement even in the short exposure to urea or the peculiar conditions of precipitation lead to a more insoluble form.

SUMMARY

Suspensions of regular crystalline insulin or of insulin OU (formed by adding water to a concentrated urea solution of crystalline or amorphous insulin) show a significant delayed resorption when compared with suspensions of amorphous insulin or solutions of crystalline insulin administered intramuscularly to rabbits.

Acknowledgments

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LOCI OF ACTION OF THYROID HORMONE ON REPRODUCTION IN THE FEMALE GUINEA PIG[1,2]

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THE per cent of premature births is higher in strain 13 guinea pigs than in any other strains or stocks for which data are available (13, 23, 58). Analysis of the records at Kansas revealed that this high per cent of wastage occurred during the second half of pregnancy. The first loss was on day 35 but most was in the third trimester which begins day 47. This wastage toward the end of pregnancy in strain 13 females suggested that a study of these animals might lead to identification of a factor exerting its effect late in gestation. When the results from a preliminary study (27) revealed that the thyroid is involved, the desirability of looking into related problems became apparent. At what time in gestation does the thyroid hormone become important for such an action? What are the effects of a more severe hypothyroidism than was produced in an earlier study of the thyroid and gestation (46)? How are the effects of the hypothyroid state on reproduction, such as the low per cent of vaginal openings and the low incidence of heat responses (46) to be explained?

MATERIAL AND METHODS

The females were obtained from stocks of strain 13 and genetically heterogeneous animals, the latter referred to for convenience as strain T. To determine the effect of thyroxine on the high pregnancy wastage in strain 13 females, 57 animals were injected subcutaneously every third day from the day of mating with 0.1 mg. dl-thyroxine[5] dissolved in mammalian Ringer's solution. Fifty-six untreated strain 13 females of the same age served as controls.

Strain 13 females were not available for the second part of the study, consequently strain T females divided into six groups were used to ascertain if the improved performance of the thyroxine-injected strain 13 animals depended on a high level of this substance throughout pregnancy or on an action localized late in pregnancy. The first

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[5] The thyroxine was supplied by E. R. Squibb and Sons, New Brunswick, New Jersey.

group was composed of 48 animals receiving thyroxine for an average of 47 days (range 22 to 72 days) prior to mating and throughout gestation. The wide range is accounted for by the circumstance that the females could be mated only when they were found in heat during examinations started day 21 of the injection period. The second group contained 47 females receiving thyroxine from the day of mating, the third group 40 females receiving thyroxine from day 21 of gestation, the fourth group 40 females treated from day 45, the fifth group 26 females injected from day 60. The sixth group was composed of 71 untreated females.

Additional information bearing on the action of the thyroid late in pregnancy was obtained from strain T females surgically thyroidectomized 92 days prior to their use experimentally. The procedure described by Peterson, Webster, Rayner and Young (46) was employed. Forty-two days after the operation these animals were injected intraperitoneally with 400 microcuries of I^{131} in 0.5 cc. of water.[6] Forty animals survived the treatment and at autopsy were free of visible thyroid rests. The 71 females which were controls for the strain T thyroxine-treated animals were also controls for the thyroidectomized group.

Oxygen consumption and heart rate were taken as indicators of thyroid activity in the strain 13 females, and of the effectiveness of the treatment in the females given thyroxine or thyroidectomized. Measurements were made within 12 hours after mating in accordance with the procedures described elsewhere (46).

The females were mated under observation as they were found in heat. Thereafter they were examined at least once daily for vaginal openings or bleeding. As is well known, the vaginal orifice closes after mating and reopens about 16 days later if the mating was sterile. An opening different from that at estrus may occur in pregnant females about the end of the first trimester (18). Bleeding at any time is indicative of resorption of one or more or the fetuses or abortion of the litter. The per cent of sterile matings, per cent of resorptions and abortions, per cent of stillborn, average litter size, and average length of gestation were recorded.

The definition of abortion is that proposed by Goy, Hoar and Young (23) as parturition earlier than 3 standard deviations below the relevant mean gestation period. For births of 1, any delivery before day 65 is an abortion, for litters of 2 any delivery before day 64, for litters of 3 and 4 any delivery before day 63, and for litters of 5 and 6 any delivery before day 62. Individuals born dead beyond days 61 to 64, depending on the size of the litter, are regarded as stillborn.

In the last part of the experiment an effort was made to account for the low incidence of vaginal openings and heat responses in the hypothyroid female guinea pig (46). Deficiency of folliculogenesis and/or corpus luteum formation and function, or a lowered responsiveness to ovariant hormones might be involved, for all have been reported to be associated with hypothyroidism (1, 7, 15, 19, 20, 37, 39, 41, 43, 49, 50, 52, 55, 63). Data were obtained from 17 of 25 thyroidectomized females not found in heat or which would not mate, from 6 of 10 untreated females not found in heat, and from 13 females not found in heat during the period of thyroxine administration. The ovaries were removed and sectioned serially for study of the number of corpora lutea and condition of the follicles.

At least a month after ovariectomy the responsiveness of the tissues mediating mating behavior to the conditioning action of α-estradiol benzoate was studied. Fourteen thyroidectomized, 14 thyroxine-injected, and 17 untreated animals were injected subcutaneously with different amounts of α-estradiol benzoate followed 36 hours later with

[6] We are indebted to Dr. Roy R. Peterson, Department of Anatomy, Washington University School of Medicine, for the administration of I^{131}.

0.2 I.U. of progesterone.[7] They were observed for the incidence and duration of heat in accordance with the methods now being used (24).

The diet consisted of commercial rabbit pellets, oats, alfalfa hay, green vegetables and water. A temperature of 70° to 75° F was maintained throughout the year.

RESULTS

The mean rate of oxygen consumption was 58.7 cc./100 gm. body weight /hour in the strain T controls; in comparison, 79.3 in the strain T females given thyroxine prior to mating ($P < .001$), 53.3 in the thyroidectomized strain T animals ($.02 > P > .01$), and 50.0 in the untreated strain 13 females ($P < .001$). Oxygen consumption became high in the strain 13 females given thyroxine from the day of mating, but the rise to the mean rate of 83.4 cc./100 gms. body weight/hour 12 hours after parturition must be corrected downward 15 to 20 cc. for the extra-thyroidal factor operating at this time (28). Mean heart rate in the strain T controls was 243 beats /min.; in comparison, 290 in the strain T females given thyroxine prior to mating ($P < .001$), 193 in the thyroidectomized strain T animals ($P < .001$), and 214 in the untreated strain 13 females ($P < .001$). Heart rate 12 hours after parturition was 254 beats/min. in the strain 13 females given thyroxine from the time of mating. In contrast with the oxygen consumption in these animals, all the increase in heart rate can be attributed to the injected thyroxine (28). The magnitude of the differences is felt to justify the designations "hyperthyroid" for the animals given thyroxine, and "hypothyroid" for the thyroidectomized and untreated strain 13 females.

The data bearing on the relationship between the level of thyroid hormone and the fertility of the mating and gestation are summarized in Table 1. The per cent of sterile matings was lower in the untreated strain 13 and untreated strain T females than in the females given thyroxine or thyroidectomized, but the differences are not significant. The result is the same when the 89 females, from which the 80 that became pregnant and were given thyroxine beginning days 21 and 45 were obtained, are added to the group of 71 untreated strain T females. Of the matings involving the 160 females, 15 or 9.4% were sterile.

There were *no* resorptions or abortions during the first trimester. The abortions and resorptions in the second and third trimesters present a problem which cannot be resolved with certainty. Thyroxine treatment of strain T females increased the abortion rate in the females in which injections were begun before mating, at the time of mating, or 21 days after mating ($P < .001$). The increase can be attributed to the abortions in the second trimester. However, thyroxine given to strain 13 females was with-

[7] α-estradiol benzoate and progesterone were supplied by the Schering Corporation, Bloomfield, New Jersey.

out effect on the abortion rate in the second trimester and the treatment reduced ($.05 > P > .02$) rather than increased abortions in the third trimester. When the data from the two strains are combined, there was an increase in second trimester abortions ($.05 > P > .02$), but no significant increase in the third trimester or in the over-all rate. As matters stand, therefore, thyroxine administration may have increased the abortion rate in the second trimester; hypothyroidism was without any statistically significant effect in either trimester.

The per cent of stillborn in strain T females given thyroxine was not significantly different from that in the controls. By contrast, the per cent of stillborn was higher in thyroidectomized strain T females than in the con-

TABLE 1. EFFECT OF ABNORMAL THYROID LEVELS ON STERILE MATINGS AND GESTATION

Treatment	N	Sterile matings		Resorptions and abortions		Day of gestation when resorption or abortion occurred		Per cent stillborn	Average litter size	Average gestation in days
		N	%	N	%	Second trimester	Third trimester			
Untreated strain 13	56	4	7.1	11	21.2	35, 36, 36	55, 56, 58, 59, 59, 59, 60, 60	40.4	3.4	68.3
Thyroxin-treated strain 13	57	7	12.3	5	10.0	37, 41	51, 52, 59	13.6	2.8	67.1
Untreated strain T	71	6	8.5	3	4.6	no loss	54, 57, 58	12.0	3.4	68.9
Thyroxin-treated strain T										
from 47 days before mating	48	8	16.7	6	15.0	30, 34, 36	55, 59, 60	21.7	3.4	66.7
from mating	47	7	14.9	11	27.0	26, 32, 43, 45, 45, 46	52, 55, 60, 60, 61	11.1	3.4	67.1
from day 21	40	—	—	6	15.0	28	49, 50, 54, 61, 63	18.5	3.0	66.9
from day 45	40	—	—	4	10.0	no loss	50, 50, 58, 59	4.4	3.2	67.8
from day 60*	26	—	—	—	—	—	62	20.2	3.6	67.9
Strain T thyroidectomized +I^{131} before mating	15	2	13.3	0	0.0	no loss	no loss	32.4	2.9	70.8

* This group was established late in the experiment after data had been obtained for the females given thyroxin beginning day 45. The animals had not been observed since the mating, consequently the embryonic and fetal loss prior to day 60 is not known.

trols ($P < .005$). This increase in stillbirths in thyroidectomized strain T females, and the reduction in stillbirths in the strain 13 females given thyroxine ($P < .001$), is regarded as evidence for a beneficial action of thyroid hormone late in pregnancy.

It is of interest that thyroxine given to strain 13 females did not reduce the per cent of stillborn below that in the untreated strain T females. Presumably, the factor or factors responsible for the 10 to 15% loss is not a deficiency of thyroid hormone, but something which is without any known differential effect on either strain. This explanation might also account for the failure of thyroxine to reduce the per cent of stillborn in the strain T females. Presumably the level of thyroid hormone in this strain is sufficient for parturition, but it would not counteract the action by the factors responsible for the 10 to 15% loss.

Litter size was not affected by thyroxine in strain T females. No explanation can be given for the statistically significant decrease in litter size in

the strain 13 females given thyroxine ($.02 > P > .01$). The smaller litters born to the thyroidectomized females can be accounted for by the reduced number of corpora lutea in these animals, an average of 2.9 in 9 females compared with 3.6 in 9 controls.

The most consistently observed effect in the experiment was on the length of gestation (Table 1). Gestation was approximately 1 day shorter in the strain T and strain 13 females given thyroxine than in their respective controls ($P < .001$). This shortening occurred when the thyroxine was given as late as day 60. Gestation was 2 days longer in the thyroidectomized females than in the controls ($P < .001$). We conclude therefore that parturition is a locus of action of thyroid hormone. It must be added, however, that its action appears to be facilitative rather than necessary, for in no hypothyroid animal did parturition fail to occur.

As we have noted, the effort to account for the reduced per cent of thyroidectomized females displaying cyclic vaginal openings and estrous periods (46) involved a study of the ovaries and the responsiveness of the tissues mediating mating behavior to the conditioning action of α-estradiol benzoate. Although there was little difference in the per cent of vaginal openings in the thyroidectomized females (92.5%) and their controls (100%), the openings in the former group occurred at variable intervals. This is probably explained by an irregularity of follicular development and ovulation not seen in the ovaries from the controls and from the thyroxine-treated females. In 10 pairs of ovaries from the thyroidectomized animals follicular development was good in the sense that the follicles appeared to be healthy, but a generation of corpora lutea was missing in 4. In the ovaries from 9 controls no generation was missing. The condition suggests that folliculogenesis in the hypothyroid female is retarded, but after ovulation has occurred, corpus luteum development is normal. The absence of corpora lutea in the 4 females is assumed to be a consequence of their complete or almost complete involution during a longer than normal interval between ovulations.

Prior to ovariectomy only 16 of 40 thyroidectomized animals (40%) were found in heat compared with 32 of 38 controls (84%). The fact that ovulation was occurring in these animals suggested that failure of heat might be attributable to a decreased responsiveness of the tissues mediating mating behavior to estrogenic stimulation. The data summarized in Table 2 support this hypothesis. Regardless of the amount of α-estradiol benzoate, the per cent of thyroidectomized animals brought into heat was less than in the controls, heat was shorter, the interval between the injection of progesterone and the beginning of heat (latency) was longer, and there was less male-like mounting. The performance of the females injected with thyroxine and given 100 I.U. of α-estradiol benzoate may be compared with that of the untreated females and the thyroidectomized animals receiving this amount of the estrogen. Without exception the per

TABLE 2. EFFECT OF ABNORMAL THYROID LEVELS ON THE MATING BEHAVIOR OF OVARIECTOMIZED GUINEA PIGS GIVEN DIFFERENT AMOUNTS OF α-ESTRADIOL BENZOATE FOLLOWED BY 0.2 I.U. OF PROGESTERONE

Group	Amount of α-estradiol benzoate	N		Per cent response	Average latency in hours	Average duration of heat in hours	Mean number of male-like mounts
		Animals	Tests				
Thyroxin	100 I.U.	14	21	85.7	6.0	5.2	46.9
Controls	100 I.U.	17	17	76.5	7.4	4.1	23.9
Thyroid-ectomized	100 I.U.	14	14	14.3	9.0	3.5	19.5
Controls	200 I.U.	17	23	82.6	6.3	5.0	18.5
Thyroid-ectomized	200 I.U.	14	27	25.9	9.9	3.0	10.6
Controls	800 I.U.	17	17	82.4	6.5	6.1	21.8
Thyroid-ectomized	800 I.U.	14	14	28.5	9.8	3.8	4.0

cent brought into heat was higher, heat was longer, latency was shorter, and more male-like mounting was displayed.

The lowered responsiveness of the thyroidectomized females to α-estradiol benzoate as measured by their mating behavior may perhaps be independent of the effect of hypothyroidism on folliculogenesis. On the other hand, the effect on folliculogenesis could be an expression of a generally lowered sensitivity of the tissues of thyroidectomized females to hormonal stimulation (1, 15).

At the end of the experiment the thyroidectomized females were injected with thyroxine every third day for 24 days. Eleven of the 14 survived and were given 100 I.U. of α-estradiol benzoate followed by 0.2 I.U. of progesterone. Unlike the response prior to this treatment (Table 2), 8 of the 11 (73%) were brought into a heat which averaged 4.8 hours. An average of 21 male-like mounts were displayed. The result is regarded as additional evidence favoring the hypothesis that thyroid hormone is important for the response of the female to α-estradiol benzoate.

DISCUSSION

The data bearing on thyroid hormone and reproduction in the female guinea pig are clearest for the relationship they reveal between this hormone and folliculogenesis, parturition, and the responsiveness of the tissues mediating mating behavior to the conditioning action of α-estradiol benzoate. Although any relationship to events during the period from fertilization until parturition is less clear, there is no strong evidence for a harmful effect of hypo- or hyperthyroidism, unless the barely significant increased fetal loss during the second trimester within the group of strain T females given thyroxine is so construed.

This view received some support from older investigations, but for the most part such support is out of context, and often contradictory reports

make evaluation difficult. The point is illustrated by a consideration of the literature dealing with the relationship between the level of thyroid activity and the ovaries. There are claims that removal of the thyroid or its suppression with antithyroid drugs has no effect on the ovaries (4, 17, 32, 33, 35, 36, 51, 56). On the other hand, ovarian degeneration, arrested folliculogenesis, and failure of ovulation are said to have resulted from thyroidectomy (19, 20, 37, 39, 41, 43, 49, 50, 52, 55, 63). Clinically there is said to be much evidence in support of thyroid administration in ovarian failure (10), on the other hand, hypothyroid women do become pregnant (3, 44).

The irregular cycles which follow thyroidectomy (1, 5, 6, 15, 34, 36, 38, 40, 41, 48, 49, 54, 59, 63) could be a consequence of harmful effects on the ovary. In most of this work, however, the nature of the effect on the ovary was not apparent. It might have been 1) failure or partial failure of follicular development with no development of corpora lutea, 2) failure of corpus luteum formation or defective functioning after a normal folliculogenesis, or 3), as we feel from our study of the guinea pig, follicular development might have been retarded, but when the stage of the preovulatory swelling was reached, ovulation and the formation of functional corpora lutea proceeded normally. As nearly as we can tell, failure of follicular development is suggested by Scow and Simpson (52), failure of corpus luteum formation by Williams, Phelps and Burch (63), Chu (7), Soliman and Reineke (55), Gillman and Gilbert (20), and an effect restricted to folliculogenesis by Ross (49).

Equally diverse reports are found with respect to the ovarian condition following the administration of thyroxine or thyroid substance. The ovaries are described as being atrophic or exhibiting incomplete folliculogenesis (9, 11, 12, 14, 16, 29, 42, 57, 62). In other studies the ovarian condition has been reported to be essentially normal, as we have found it in the guinea pig (25, 26, 37, 60, 61).

The lack of any marked effect of abnormal levels of thyroid hormone on gestation in contradistinction to parturition is to be contrasted with the numerous reports of resorptions and abortions in laboratory and domestic mammals given thyroxine or thyroid substances (11, 14, 21, 29, 37, 60, 62) or made hypothyroid by thyroidectomy or the administration of antithyroid drugs (2, 8, 32, 36, 43, 49, 51). There is however supporting evidence for the view that pregnancy may proceed normally when thyroid substances are administered (53) or following surgical or chemical thyroidectomy (4, 17, 22, 30, 33, 47, 56). Clinically the evidence is considered substantial that hypothyroidism is a contributing factor to abortions (31, 45), but a careful reading of the latter articles and others already reviewed (46) reveals that reservations are still necessary.

We cannot account for the different results and opinions. Species differences may be involved and the suggestion made following a study of

the male guinea pig (64), that from species to species the range of thyroid activity compatible with reproduction differs, could be extended to the female. Despite the possible importance of species differences, the guinea pig was used in a number of the investigations cited above (11, 14, 21, 29, 53), consequently some explanation other than differences between species must be sought.

We have thought of methodology. The present study appears to be the first in which the time of mating was recorded accurately, in which the animals were examined daily for signs of resorption or abortion, in which a criterion for abortion in contradistinction to stillbirth was established, and in which the level of thyroid activity and the effectiveness of exogenous thyroxine were estimated from measurements of oxygen consumption and heart rate. The differences between the results reported by Peterson, Webster, Rayner and Young (46) and those obtained in this study, chiefly in the per cent of fertile matings, are attributed to the variation which would be expected between the samples and to refinements in procedure. In that work, as in this, the low incidence of estrus and the high rate of stillbirths were characteristic of hypothyroid animals.

The relationship of the thyroid to parturition is one of the most definite we have established. There are reports that pregnancy is prolonged in hypothyroid females (8, 36, 43, 47), but the early delivery of living young to females given thyroxine or thyroid substance does not seem to have been reported, nor has the suggestion been made for any species that the thyroid hormone facilitates parturition and need be present only late in gestation in order to exert its effect.

SUMMARY AND CONCLUSIONS

Loci of action of the thyroid hormone on reproduction in the female guinea pig were investigated by the use of an inbred strain of hypothyroid animals injected with thyroxine, and by the use of genetically heterogeneous females in which oxygen consumption and heart rate were altered by injections of thyroxine and by thyroidectomy followed by I^{131}. After the females had been mated with normal males, the fertility of the mating was determined and gestation was followed by daily examination of the vaginal orifice for signs of resorption and abortion. Ovaries were removed from the animals not found in heat and studied for abnormalities of follicular development and corpus luteum formation. The responsiveness of the tissues mediating mating behavior to the conditioning action of α-estradiol benzoate was tested.

The per cent of matings that were fertile was not significantly different in any group of females. There were no resorptions or abortions during the first trimester of pregnancy. The administration of thyroxine may have increased the abortion rate in the second trimester, but hypothyroidism appears to have been without effect in the second and third trimesters.

Gestation was approximately 1 day shorter in strain T and strain 13 females given thyroxine than in their respective controls ($P < .001$). In the thyroidectomized females it was 2 days longer than in the controls ($P < .001$). Thyroxine injections had no effect on the stillbirth rate of the strain T females but did reduce that of the strain 13 females ($P < .001$). The stillbirth rate in the thyroidectomized females was higher than in the controls ($P < .005$). These effects are the basis for the conclusion that parturition is a locus of action of thyroid hormone.

The reduced per cent of thyroidectomized females displaying cyclic vaginal openings and estrus indicates that a second locus of action is the events leading to ovulation and estrus. The effect is accounted for 1) by the lowered responsiveness of the tissues mediating mating behavior to the conditioning action of α-estradiol benzoate and 2) by a retardation of follicular development. However, when the stage of the preovulatory swelling is reached, ovulation and the formation of functional corpora lutea appear to proceed normally.

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DISTRIBUTION OF INJECTED CORTISOL-4-C^{14} IN NORMAL AND SHOCKED RATS

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THE mechanism by which cortisol or any other adrenocortical hormone fulfills its physiological function is unknown. It appears reasonable to assume that the active principle is being attached to the protein structure of the target tissue prior to its specific action; such possible combinations with protoplasmic receptors have been discussed for steroid hormones in general (1). Labeling with radiocarbon or tritium has been used to follow in the rodent the metabolic fate of testosterone (2), progesterone (2, 3, 4), cortisone (5, 6) and cortisol (7). In most of these studies the steroid doses administered exceeded physiological concentrations and the time periods investigated were generally of the order of hours or days.

The present study was undertaken in order to elucidate the fate of cortisol-4-C^{14} during the first hour after administration. The doses given were near the physiological level of the 17-hydroxycorticosteroids (8). The first phase of this investigation is concerned with the distribution of cortisol or its metabolites in normal rats and in rats subjected to tourniquet shock.

EXPERIMENTAL

Preparation of Tissue Samples. Male Sprague-Dawley rats were used after fasting overnight. During the experiments they were kept without access to food or water, in cages which permitted the separation of urine from feces. In the normal control series, 10 to 15 μg. of cortisol-4-C^{14}* dissolved in 1.0 to 1.5 ml. saline containing 10% propylene glycol were injected into the heart with a size 21 needle. The specific activity of the cortisol used was 4.08 μc per mg., 10 μg. corresponding to about 40,000 counts per minute as determined by our counting technique. The radiochemical purity was found to be higher than 94% (9). In a second series of control animals, normal rats were injected

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with a mixture of 74 μg. of corticosterone[4] and 16.5 μg. of cortisol-4-C^{14} in 1.5 ml. saline containing 10% propylene glycol. Five, 15 or 60 minutes after the injection, the rats were lightly anesthetized with ether and the thoracic cavity was opened. Approximately 2 mg. of heparin in 0.5 ml. saline were injected into the heart with a size 18 needle. A few seconds later, the blood was withdrawn from the heart as completely as possible, stored in the refrigerator overnight and the plasma prepared by centrifugation. No radioactivity was observed in the washed red cells of the normal or stressed rats.

For the production of tourniquet shock (10), the teeth of the starved rats were clipped after a light ether anesthesia, and five turns of No. 30 Eberhard Faber-rubber bands were placed unilaterally on one forelimb and one hindlimb for $4\frac{1}{2}$ hours. The rubber bands were then cut and completely removed. After a period of one hour, the animals were injected with cortisol-4-C^{14}. The injection and all the following procedures were performed as outlined for the normal rats. Other details may be found in the tables. For brevity, the animals subjected to tourniquet shock will be referred to as "stressed" rats.

Immediately after the blood was drawn from the heart, the liver was perfused *in situ* through the hepatic portal vein, with 30 ml. of saline containing 2 mg. heparin. The rinsing fluid was discarded. The organs listed in the tables were removed, their wet weight determined, and then they were dried in an oven at 80° C for 3–4 days. Some fat tissue adhering to the organs was left in place. Since a complete separation of the muscles from the bones takes too much time to be practical, the bone structure was left with the muscle fraction during the preparation of the counting solution (11). This procedure was found to be justified since repeated experiments showed that under the conditions of our studies the bones, completely freed of adhering tissue, did not contain any measurable amounts of C^{14}. They are insoluble in the formamide used as a solvent (see below).

The gastro-intestinal tract was processed with its contents included. Scrotum and epididymis were left connected to the testes. The skin area of injection was tested separately and is listed in Tables 1 and 2 as "skin (inj. site)". The data given for the bladder are inclusive of the urine content. Expired carbon dioxide was not collected since preliminary experiments failed to demonstrate measurable radioactivity in the expired CO_2. This is in agreement with the finding of Wyngaarden, Peterson and Wolff (7) who could not detect C^{14} in the expired CO_2 of rats during 96 hrs. after a dose of cortisol-4-C^{14} which was more than 7 times higher than those used in the present studies. In the rats subjected to tourniquet shock, the occluded limbs were cut off, dried and separately analyzed for radioactivity.

Determination of C^{14}. In a number of preliminary experiments, C^{14} was estimated by wet combustion (12) of either organ extracts or by direct combustion of the small organs. However, the time required for the analysis of many organs in a large number of animals made it virtually impossible to use this procedure. Therefore, a direct plating method was developed which permits a comparatively rapid determination of C^{14}-containing compounds in animal tissues. The dried tissues or organs were dissolved in hot formamide and the colloidal solutions plated on aluminum planchets. Details of this technique including self-absorption curves have been given in a previous report (11). The solutions were applied on the planchets without the use of lens paper (13).

The planchets were counted with a Q-gas flow counter[5] or in an automatic gas flow counter.[6] The counts per minute (cpm.) were corrected for background and for small daily variations in the response of the counters by referring to a standard polystyrene

[4] The authors are indebted to The Upjohn Company, Kalamazoo, Michigan, for supplying this material (Batch No. 110-MHK-10).

[5] Model D46, Nuclear Instrument and Chemical Corporation, Chicago 10, Ill.

[6] Model SC-50, Tracerlab, Inc., Boston 10, Massachusetts.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN NORMAL RATS 5 MINUTES AFTER INJECTION OF 58,000 CPM OF CORTISOL-4-C^{14}, IN PER CENT OF TOTAL COUNTS INJECTED

Rat No.	N1	N2	N3	N4	N5
Weight in gm.	300	330	335	320	330
Plasma	4.8	6.0	6.1	7.3	9.1
Muscle	51.7	56.4	43.5	46.1	38.3
Liver	24.9	26.4	24.1	24.5	26.4
GI Tract	9.9	15.2	12.9	9.1	11.9
Kidneys	2.9	3.5	2.8	4.4	5.1
Thymus	0.3	0.1	0.2	0.1	0.2
Spleen	0.3	0.2	0.2	0.2	0.3
Lungs	2.2	0.9	0.2	1.5	1.4
Heart	0.3	0.5	0.4	0.8	0.3
Pancreas	0.9	1.4	0.4	0.4	0.8
Brain	0.0	0.1	0.3	0.1	0.4
Pituitary	0.01	0.01	0.01	0.00	0.01
Adrenals	0.06	0.03	0.03	0.03	0.05
Thyroid	0.02	0.01	0.00	0.01	0.02
Testes	0.2	0.0	0.8	0.6	0.3
Seminal Vesicles	0.3	0.3	0.4	0.0	0.3
Prostate	0.1	0.1	0.2	0.2	0.3
Bladder	0.06	0.05	0.08	0.11	0.06
Skin	0.0	8.6	0.0	8.4	0.0
Skin (inj. site)	0.0	2.3	0.1	0.4	1.2
Total cpm Recovered	57,400	70,900	53,700	60,500	55,900
Total % Recovery	98.9	122.1	92.6	104.2	96.4

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN STRESSED RATS 5 MINUTES AFTER INJECTION OF CORTISOL-4-C^{14}, IN PER CENT OF TOTAL COUNTS INJECTED

Rat No.	S1	S2	S3	S4	S5
Weight in gm.	330	310	325	310	325
Cpm injected	53,380	49,900	53,380	49,900	51,640
Plasma	11.1	7.8	13.2	6.1	8.3
Muscle (total	53.7	48.8	41.5	40.5	45.3
Muscle, exclusive of limbs	45.9	42.6	39.8	34.0	36.8
Non-Occluded Limbs	2.2	3.5	1.7	3.7	4.6
Occluded Limbs	5.6	2.7	0.0	2.8	3.9
Liver	44.1*	22.9	26.8	25.9	29.2
GI Tract	12.7	12.0	9.6	10.6	11.2
Kidneys	4.4	2.5	2.9	2.7	3.9
Thymus	0.5	0.3	1.0	0.5	0.6
Spleen	0.2	0.3	0.4	0.1	0.3
Lungs	1.5	2.2	3.8	2.4	3.1
Heart	1.0	0.8	1.1	0.7	0.6
Pancreas	1.5	0.5	0.5	0.3	0.5
Brain	0.1	0.3	0.2	0.3	0.5
Pituitary	0.01	0.01	0.01	0.02	0.01
Adrenals	0.07	0.09	0.1	0.04	0.06
Thyroid	0.02	0.02	0.02	0.02	0.03
Testes	0.2	1.1	0.3	1.0	0.2
Seminal Vesicles	0.3	0.3	0.4	0.4	0.2
Prostate	0.1	0.2	0.2	0.1	0.2
Bladder	0.05	0.05	0.05	0.07	0.03
Skin	2.8	8.4	9.3	0.0	3.9
Skin (inj. site)	1.1	0.8	1.0	2.7	0.0
Lung Clot	—	—	0.2	—	—
Total cpm Recovered	72,400	54,600	60,100	47,200	55,700
Total % Recovery	135.7	109.4	112.5	94.5	107.9

* Value obviously incorrect.

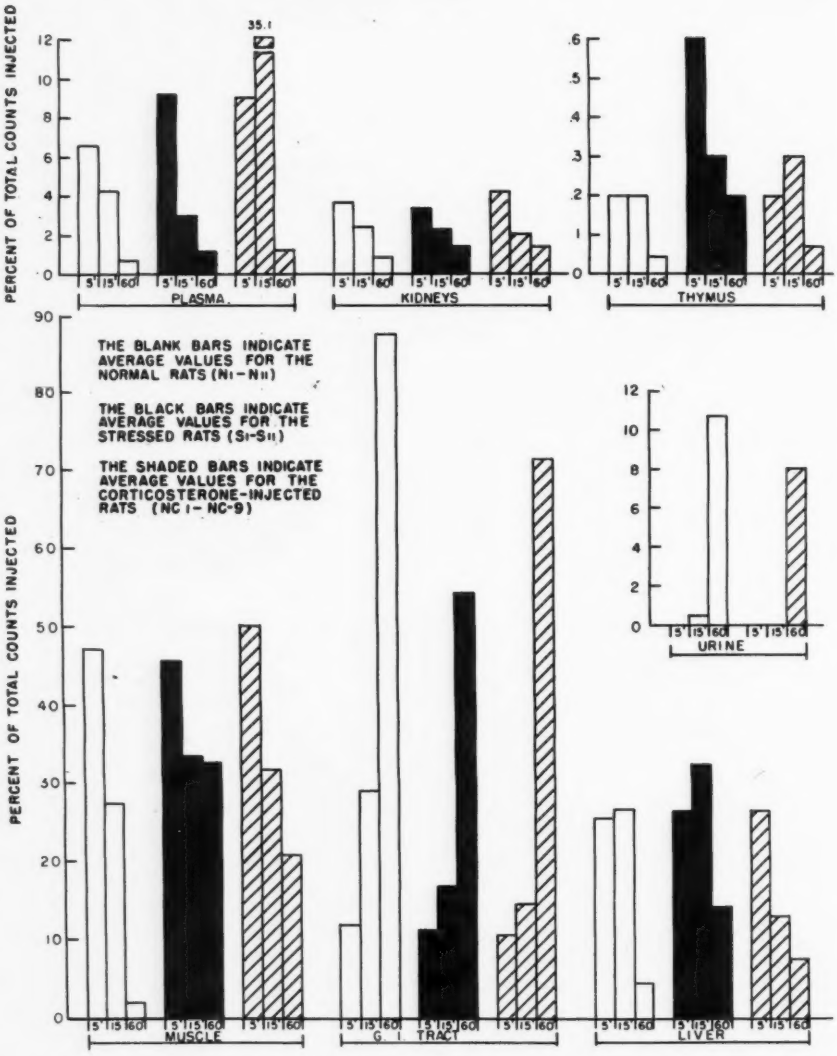
PERCENT OF TOTAL COUNTS INJECTED
35.1
PLASMA
KIDNEYS
THYMUS
THE BLANK BARS INDICATE AVERAGE VALUES FOR THE NORMAL RATS (N I - N II)
THE BLACK BARS INDICATE AVERAGE VALUES FOR THE STRESSED RATS (S I-S II)
THE SHADED BARS INDICATE AVERAGE VALUES FOR THE CORTICOSTERONE-INJECTED RATS (NC I - NC-9)
URINE
MUSCLE
G. I. TRACT
LIVER
5' 15' 60'

FIG. 1. Distribution of radioactivity (per cent of total counts injected) at 5, 15 and 60 minutes after injection of cortisol-4-C^{14}, in normal, stressed and corticosterone-injected rats.

source labeled with C^{14}.† The number of counts measured gave a standard deviation of counting rate between ± 3 and $\pm 10\%$ (11).

The cpm values obtained were corrected for self absorption (11), calculated for the total dry weight of the various organs and expressed as per cent of the injected dose

† Obtained from Oak Ridge National Laboratory on allocation from the Isotope Division, U. S. Atomic Energy Commission.

(Tables 1, 2, 6, 8, 10; Fig. 1). In addition, C^{14} content was expressed as specific activity i.e., cpm per gm. of the dry tissue or per ml. of plasma or urine (Tables 3, 4, 7, 9, 10; Fig. 2). In the 5-minute experiments, the dry weights of the organs were not determined directly but computed from the data of Table 5. The total amount of plasma for the individual animals was calculated according to Berlin, Huff, VanDyke and Hennessy (14).

RESULTS AND DISCUSSION

In contrast to most other species, corticosterone appears to be the main corticosteroid in the rat (15). Cortisol could not be detected in the urine from albino rats (16); however, small quantities were found in adrenal venous blood (17). The capacity of rat adrenal tissue to produce cortisol *in vitro* has also been demonstrated (18, 19). It has been established that

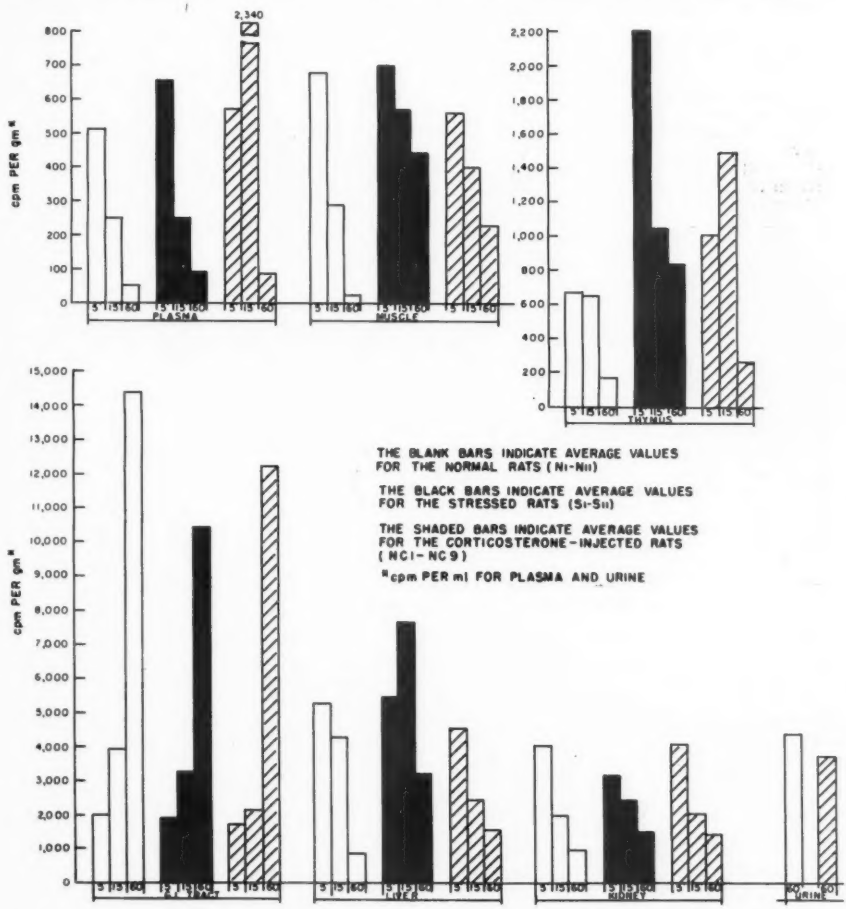
cpm PER gm*
2,340
PLASMA
MUSCLE
THYMUS
LIVER
KIDNEY
URINE
5 15 60
THE BLANK BARS INDICATE AVERAGE VALUES FOR THE NORMAL RATS (N1-N11)
THE BLACK BARS INDICATE AVERAGE VALUES FOR THE STRESSED RATS (S1-S11)
THE SHADED BARS INDICATE AVERAGE VALUES FOR THE CORTICOSTERONE-INJECTED RATS (NC1-NC9)
*cpm PER ml FOR PLASMA AND URINE

FIG. 2. Specific activity (cpm. per gm. of dry weight) at 5, 15 and 60 minutes after injection of cortisol-4-C^{14}, in normal, stressed and corticosterone-injected rats.

TABLE 3. SPECIFIC ACTIVITY (CPM PER GM. OF DRY WEIGHT) OF VARIOUS ORGANS IN NORMAL RATS 5 MINUTES AFTER INJECTION OF CORTISOL-4-C^{14}

Rat. No.	N1	N2	N3	N4	N5
Plasma*	400	460	460	570	700
Muscle	760	760	580	780	510
Liver	5,600	5,390	4,860	5,160	5,400
GI Tract	1,830	2,580	2,130	1,580	1,990
Kidneys	3,310	3,640	2,880	4,690	5,320
Thymus	1,190	360	640	520	650

* Cpm per ml.

TABLE 4. SPECIFIC ACTIVITY (CPM PER GM. OF DRY WEIGHT) OF VARIOUS ORGANS IN STRESSED RATS 5 MINUTES AFTER INJECTION OF CORTISOL-4-C^{14}

Rat No.	S1	S2	S3	S4	S5
Plasma*	780	550	950	430	570
Muscle, exclusive of Limbs	810	750	710	600	640
Non-Occluded Limbs	190	290	150	320	380
Occluded Limbs	450	220	—	230	310
Liver	9,160	4,730	5,630	5,330	6,040
GI Tract	2,190	2,060	1,670	1,820	1,930
Kidneys	4,210	2,340	2,780	2,510	3,720
Thymus	2,000	1,180	3,850	1,990	2,060

* Cpm per ml.

TABLE 5. DRY WEIGHT OF ORGANS IN PER CENT OF TOTAL BODY WEIGHT

Normal Rats

Rat No.	N6	N7	N8	N9	N10	N11	Avg.	S.D.±
Weight in gm.	299	294	300	320	320	347	314	—
Muscle	12.6	12.9	12.6	13.0	14.0	13.1	13.1	0.59
Liver	0.84	0.78	0.86	0.83	0.96	0.89	0.86	0.06
GI Tract	0.90	1.05	0.96	1.22	1.04	1.04	1.05	0.11
Kidneys	0.17	0.18	0.17	0.14	0.18	0.18	0.17	0.016
Thymus	0.028	0.037	0.068	0.040	0.040	0.036	0.042	0.014
Total							15.2	

Stressed Rats

Rat No.	S6	S7	S8	S9	S10	S11	Avg.	S.D.±
Weight in gm.	322	321	314	330	346	325	326	—
Muscle (Total)	12.4	12.8	13.2	13.1	13.6	13.8	13.1	—
Muscle, exclusive of limbs	8.1	8.6	9.0	9.6	9.9	9.8	9.2	0.85
Non-occluded Limbs	2.0	2.2	2.0	1.8	1.8	1.8	1.9	0.21
Occluded Limbs	2.3	2.0	2.2	1.7	1.9	2.2	2.0	0.27
Liver	0.74	0.82	0.80	0.80	0.78	0.76	0.78	0.03
GI Tract	0.99	0.95	0.93	1.02	0.91	0.87	0.94	0.055
Kidneys	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.00
Thymus	0.048	0.063	0.036	0.041	0.046	0.030	0.444	0.011
Total							15.0	

TABLE 6. DISTRIBUTION OF RADIOACTIVITY IN NORMAL (N6–N8) AND STRESSED (S6–S8) RATS, 15 MINUTES AFTER INJECTION OF CORTISOL-4-C^{14}, IN PER CENT OF TOTAL COUNTS INJECTED

Rat No.	N6	N7	N8	S6	S7	S8
Weight in gm.	299	294	300	322	321	314
Cpm injected	39,500	39,500	39,500	59,300	59,300	59,300
Plasma	2.9	3.2	6.8	3.1	2.9	3.2
Muscle (Total)	28.5	20.0	33.6	38.5	31.2	30.7
Muscle, exclusive of limbs	—	—	—	29.7	25.2	23.1
Non-occluded Limbs	—	—	—	4.0	2.1	2.3
Occluded Limbs	—	—	—	4.8	3.9	5.3
Liver	26.1	24.2	29.5	27.6	31.8	37.1
GI Tract	31.8	36.9	18.3	14.9	16.0	19.4
Kidneys	2.8	2.8	2.0	1.7	3.3	2.0
Thymus	0.1	0.2	0.4	0.3	0.3	0.2
Urine	—	0.4	—	—	—	—
Total cpm Recovered	36,400	34,600	35,900	51,100	50,600	54,900
Total % Recovery	92.2	87.7	90.6	86.1	85.5	92.6

TABLE 7. SPECIFIC ACTIVITY (CPM PER GM. OF DRY WEIGHT) OF VARIOUS ORGANS IN NORMAL (N6–N8) AND STRESSED (S6–S8) RATS, 15 MINUTES AFTER INJECTION OF CORTISOL-4-C^{14}

Rat No.	N6	N7	N8	S6	S7	S8
Plasma*	170	190	390	250	240	270
Muscle†	300	210	350	680	540	490
Non-Occluded Limbs	—	—	—	370	180	210
Occluded Limbs	—	—	—	390	360	460
Liver	4,110	4,120	4,540	6,910	7,170	8,770
GI Tract	4,650	4,730	2,530	2,780	3,110	3,960
Kidneys	2,100	2,120	1,600	1,750	3,500	2,200
Thymus	460	670	820	1,200	770	1,150

* Cpm per ml.
† Total muscle for rats N6–N8; muscle exclusive of limbs for rats S6–S8.

TABLE 8. DISTRIBUTION OF RADIOACTIVITY IN NORMAL (N9–N11) AND STRESSED (S9–S11) RATS 60 MINUTES AFTER INJECTION OF 59,300 CPM OF CORTISOL-4-C^{14}, IN PER CENT OF TOTAL COUNTS INJECTED

Rat No.	N9	N10	N11	S9	S10	S11
Weight in gm.	320	320	347	330	346	325
Plasma	0.9	0.7	0.5	1.5	0.8	1.3
Muscle (Total)	3.7	2.0	0.0	36.9	32.0	28.8
Muscle, exclusive of Limbs	—	—	—	29.1	25.0	18.3
Non-Occluded Limbs	—	—	—	3.9	3.6	5.4
Occluded Limbs	—	—	—	3.9	3.4	5.1
Liver	5.6	4.4	2.8	16.6	12.5	12.2
GI Tract	85.6	88.5	87.7	42.5	57.8	61.9
Kidneys	1.1	0.7	0.5	1.5	1.3	1.4
Thymus	0.03	0.05	0.03	0.3	0.1	0.1
Urine	7.5	9.7	15.0	—	—	—
Total cpm Recovered	61,800	63,000	63,100	58,900	62,000	63,000
Total % Recovery	104.4	106.1	106.5	99.3	104.5	106.3

TABLE 9. SPECIFIC ACTIVITY (CPM PER GM. OF DRY WEIGHT) OF VARIOUS ORGANS IN NORMAL (N9–N11) AND STRESSED (S9–S11) RATS, 60 MINUTES AFTER INJECTION OF CORTISOL-4-C^{14}

Rat No.	N9	N10	N11	S9	S10	S11
Plasma*	76	56	34	120	58	100
Muscle†	56	27	0	540	430	340
Non-Occluded Limbs	—	—	—	390	360	540
Occluded Limbs	—	—	—	420	320	430
Liver	1,250	860	530	3,740	2,760	3,070
GI Tract	13,000	15,800	14,400	7,510	10,800	12,900
Kidneys	1,480	690	560	1,520	1,270	1,530
Thymus	160	230	130	1,460	330	700
Urine*	4,440	4,440	4,440	—	—	—

* Cpm per ml. The urine collected from rats N9–N11 was pooled.
† Total muscle for rats N9–N11; muscle exclusive of limbs for rats S9–S11.

TABLE 10. DISTRIBUTION OF RADIOACTIVITY (PER CENT OF TOTAL COUNTS INJECTED) AND SPECIFIC ACTIVITY (CPM PER GM. OF DRY WEIGHT) FOR NORMAL RATS, 5, 15, AND 60 MINUTES AFTER INJECTION OF 66,300 CPM. OF CORTISOL-4-C^{14} PLUS 74 μg. OF CORTICOSTERONE

	Distribution of radioactivity			Specific activity		
A. 5 Minutes After Injection						
Rat No. Weight in gm.	NC1 445	NC2 478	NC3 467	NC1	NC2	NC3
Plasma*	13.6	6.9	6.7	890	410	420
Muscle	44.8	51.5	53.4	500	580	590
Liver	16.3	22.6	26.1	3,920	4,300	5,420
GI Tract	9.0	9.6	12.0	1,550	1,470	2,070
Kidneys	3.7	4.7	4.3	3,910	4,180	4,270
Thymus	0.3	0.2	0.2	1,390	690	950
Total cpm Recovered	58,200	63,300	68,200			
Total % Recovery	87.7	95.5	102.7			
B. 15 Minutes After Injection						
Rat No. Weight in gm.	NC4 475	NC5 413	NC6 447	NC4	NC5	NC6
Plasma*	4.6	43.2	57.6	280	3,010	3,710
Muscle	47.3	34.9	22.3	530	420	250
Liver	18.7	12.9	6.0	3,440	3,630	1,200
GI Tract	29.6	8.3	4.9	4,320	1,330	660
Kidneys	2.9	2.5	1.0	2,670	2,440	1,070
Thymus	0.1	0.5	0.3	580	2,690	1,260
Total cpm Recovered	68,500	67,800	61,100			
Total % Recovery	103.2	102.3	92.1			
C. 60 Minutes After Injection						
Rat No. Weight in gm.	NC7 452	NC8 457	NC9 425	NC7	NC8	NC9
Plasma*	1.4	1.1	1.5	90	70	100
Muscle	27.0	13.7	21.3	300	150	230
Liver	7.3	6.9	7.6	1,390	1,500	1,690
GI Tract	64.0	79.5	74.7	8,610	14,800	13,200
Kidneys	1.6	1.1	1.7	1,510	1,090	1,670
Thymus	0.05	0.05	0.10	210	180	420
Urine*	1.1	10.3	13.0	3,750	3,750	3,750
Total cpm Recovered	68,000	74,800	79,500			
Total % Recovery	102.5	112.6	119.9			

* Cpm per ml.

cortisol is metabolically transformed and excreted in the rat (**7**). The amounts of cortisol-4-C^{14} administered in the present studies were near the physiological peripheral level of 17-hydroxycorticosteroids in the normal rat which have been determined to be of the order of 0.85 μg. (8) or 0.7 μg. (20) per 10 ml. If the total amount of cortisol-4-C^{14} injected would be immediately distributed throughout the blood the maximal concentration would be about 10-times the peripheral level mentioned above. The intracardial injection under the conditions described is not expected to increase the blood concentration above the levels of the adrenal venous blood which can be assumed to contain at least 10 times as much as the peripheral blood if an analogy can be made to the observations in the dog (21, 22) and human (23).

In the present investigation it was observed that 5 minutes after cortisol administration only 6.7% of the injected dose was present in the blood of normal rats (Table 1, Fig. 1). This corresponds to a concentration of the injected cortisol of about 0.8 μg. per 10 ml. of plasma, the physiological level for 17-hydroxycorticoids. The distribution of the injected cortisol-4-C^{14} or its metabolites in the normal and stressed rats after 5, 15 and 60 min. is given in per cent of the administered dose in Tables 1, 2, 6, 8 and in Figure 1. Tables 3, 4, 7, 9 and Figure 2 show the specific activity per unit weight of the organs for a comparison of the actual C^{14} concentrations in the various organs and tissues. It appears noteworthy that 5 minutes after injection almost 50% of the radioactive material was found in the muscles (Tables 1 and 2). A comparison with the plasma values shows that this was a retention by the muscle tissue rather than the result of the presence of residual blood: the total plasma after the same time period contained only about 7% of the injected dose in the normal animals, and 9% in the stressed rats. The specific activity in the muscle was not higher than in other tissues, indicating that no particular concentration took place. The present findings substantiate the observations of Tomizawa, Narahara, Gibbons and Williams (20) who concluded from experiments in hepatectomized rats that the initial decline of the 17-hydroxycorticosteroid level after intravenous injection of cortisol was due to a rapid distribution of the hormone throughout the body tissues.

In the normal animals, the cortisol-C^{14} activity was gradually released from the muscles so that at 60 min. after administration only about 2% of the injected dose was left (Table 8). This material was obviously shifted through plasma and liver to the gastrointestinal tract as indicated by the changes in the percentage distribution (see Fig. 1). In the stressed rat, however, this rapid clearance of the C^{14}-compounds from the muscle did not take place; the percentage of activity found after 60 min. was practically the same as that after 15 min. The seemingly obvious explanation that the delayed clearance was caused by the decreased circulation in the stressed animal (24) is not considered probable since the plasma values in the stressed rats after 15 and 60 min. were not higher than those in the

normal animals. On the basis of the known dissolving and binding capacity of blood for cortisol (25), higher plasma concentrations could be expected after 60 min. if the delay in the muscle clearance was caused only by retarded circulation. Furthermore, the stressed rats had a lower plasma activity after 60 min. than after 15 min. in contrast to the muscle activity which showed no change.

In an attempt to account for a possible influence of the increased endogenous corticosteroid production on the retention of the radioactive material in the skeletal muscle of the stressed animals, a control series was prepared in which the corticosteroid level was increased. Since corticosterone appears to be the predominant corticoid in the rat (15), normal rats were injected with cortisol-4-C^{14} in addition to about 5-times as much corticosterone. The results (Figs. 1 and 2, Table 10) show that the turnover of the C^{14} compound(s) in the muscles was markedly delayed by the higher corticosterone level. Percentage distribution and specific activity in the skeletal muscle of the corticosterone-injected animals approach the values obtained in the stressed rats.

The chemical nature of the radioactive material will be discussed in a later report. The C^{14} content of the occluded and non-occluded legs did not show consistent differences. No explanation can be given for the comparatively high plasma activity value in the corticosterone-injected rats after 15 min.

The C^{14}-values of the liver (Fig. 1) reflect the changes observed in other organs: they decrease in the normal animals as the activity of the muscles diminishes, but in the stressed rats they decrease at a slower rate, in accordance with the delayed clearance in the muscles. The gradual excretion into the gastro-intestinal tract is illustrated by the stepwise increase in its radiocarbon content. The delayed turnover of the cortisol-4-C^{14} in the stressed animal is obvious from the slower activity increase in the gastrointestinal tract, apparently caused by the decreased circulation as well as the retention of the active material in the muscles. Again the activity values in the corticosterone-injected rats after 60 min. are intermediate between the two other groups.

It has been established by several authors that the rat excretes cortisol metabolites predominantly in the feces (7, 20, 21, 26). In the present studies, 87% of the material injected into normal rats was found in the gastrointestinal tract after 60 min. whereas the urine contained only 11% after the same time interval. These figures indicate a considerably faster biliary elimination than the 35–50% observed by Wyngaarden, Peterson and Wolff (7) after 1 hr. The explanation for this difference may be that Wyngaarden *et al.* administered the cortisol by subcutaneous injection, and that the dose was approximately 6 times that given in the present study.

The ratio of 8:1 for the activity in the gastrointestinal tract and the

urine after 60 min. (Table 8, Fig. 1) is higher than that of 7:3 observed by Wyngaarden *et al.* (7) after 4 days. Obviously part of the radiocarbon material, initially excreted *via* bile, later enters the enterohepatic circulation and appears in the urine. No urine could be collected from the stressed rats. The activities measured in the kidneys reflect the concentrations in the plasma.

It appears noteworthy that the percentage distribution of the cortisol-C^{14} material in the thymus was found to be higher in the stressed rats than in the controls. The specific activity of the thymus of the stressed animals also exceeded that of the normal rats. This result appears of interest in view of the marked effect of the glucocorticosteroids on thymus involution (27, 28).

In conclusion, the present study shows that injected cortisol undergoes a rapid turnover in the rat. The bulk of the material appears to become attached to the skeletal muscle from where it is being gradually released through the liver into excretory pathways. This retention in the muscles is prolonged in the rat subjected to tourniquet shock. However, the delayed release in the stressed animal does not seem to be caused by a mechanism of steroid-receptor attachment specific for the condition of shock. This is indicated by the observation of a similar delayed turnover in normal rats in which the corticoid level was increased by injection of corticosterone. The capacity of the liver to metabolize the steroid may be the limiting factor in the turnover rate observed in the skeletal muscle, or the level of the corticoids in the muscle may regulate their release.

SUMMARY

Small amounts of cortisol-4-C^{14} were injected intracardially into rats subjected to tourniquet shock and into normal controls. The radioactivity of various organs was determined at 5, 15 and 60 min. intervals after injection. After 5 min. almost 50% of the administered C^{14} material was found in the skeletal muscle. In the normal animals it was rapidly released into excretory pathways so that 60 min. after injection only 2% remained in the muscular tissue. In the stressed rats, however, the muscle clearance was delayed so that about 33% was still present after the 60-min. interval. In normal rats injected with corticosterone in addition to cortisol-4-C^{14} a retarded turnover of the radioactive material was also observed. The significance of the findings is discussed.

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"TOTAL" PANCREATECTOMY IN THE RAT: OPERATION, EFFECTS, AND POSTOPERATIVE CARE

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TOTAL extirpation of the pancreas in the dog and the cat results in the immediate onset of severe diabetes and marked impairment of the intestinal absorption of food (1, 2, 3). If not treated with insulin, they die within a few days in diabetic coma.

Total pancreatectomy in the rat is difficult because of certain anatomical peculiarities of the pancreas in this species. The gland extends over a large area and has numerous pancreatic ducts that empty directly into the common bile duct (4). Although several methods have been described for extirpating the rat's pancreas (5, 6, 7, 8, 9, 10, 11), only one author (8) has reported the immediate onset of diabetes after pancreatectomy; none have described impaired absorption of food. Many of the workers reported that the operated rats survived for several months in good health without being treated with insulin. Because of these results it has been generally concluded that the rat does not become as severely or as quickly diabetic after pancreatectomy as do other species (12).

This paper describes the effects of removing at least 99.5% of the pancreas of the rat. Within 18 hours after the operation all rats had hyperglycemia, glycosuria, ketonuria, fatty livers, and large amounts of intracellular fat in the renal tubules. If not treated with insulin, all died within 48 hours. For control of the diabetes they required large amounts of insulin (2.5 units per gram of food). In addition to the severe diabetes these operated rats had markedly impaired intestinal absorption and consequently needed pancreatin.

EXPERIMENTAL

Female rats weighing 110–150 gm. of the Instituto de Biologia y Medicina Experimental were used in this study. Prior to pancreatectomy they were either fed the common diet of the institute (bread, milk, corn, wheat, cod liver oil, and alfalfa greens) or diet #4370 (Table 1). If the latter is made with finely ground casein, fine enough to

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pass through a 100 mesh sieve,[2] it can be made into a suspension which can be easily fed through small polyethylene tubing.

The blood glucose concentration was determined, in samples taken from the tail, by the method of Haslewood and Strookman (13). The amount of nitrogen in the urine, food, and feces was measured by the micro-Kjeldahl method; urinary glucose by Benedict's quantitative method (14); and urinary ketone bodies by the nitroprusside (Rothera's) test or the quantitative method of Van Slyke (15).

Surgical Anatomy: Although the gross anatomy of the pancreas in the rat has been discussed in several articles on pancreatectomy (5, 6, 7, 8, 9, 10, 11), none have described the numerous pancreatic ducts and their relationship to the bile duct and to the pancreas (4). The rat pancreas has 2 large and 8–15 small ducts which empty into the bile

TABLE 1. DIET #4370

Casein-vitamin free	20.0%
l-cystine	0.3
Dextrin	66.7
Oil (corn)	8.0
Salt (HM&W)[a]	4.0
Vitamin powder S-1[b]	1.0
Vitamin A 55,000 USP units/gm. Vitamin D 11,000 USP units/gm.[c]	0.3 gm./kg. of diet
Vitamin E	50 mg./kg. of diet

[a] Hubbel, Mendel and Wakeman, *J. Nutrition* 14: 273. 1937.

[b] Vitamin powder S-1:

	100 gm.
Thiamine	40 mg.
Riboflavin	60 mg.
Pyridoxin	50 mg.
Calcium pantothenate	400 mg.
Nicotinic acid	400 mg.
Choline	20,000 mg.
Inositol	2,000 mg.
Biotin powder*	200 mg.
Folic acid	20 mg.
Vitamin K	20 mg.
Starch (Argo)	77,210 mg.

* *Biotin powder* is a mixture of 10 mg. biotin in one gram of starch

[c] Natola (Parke, Davis & Co.).

duct; none enter the duodenum directly. For operative purposes the pancreas can be divided into 3 parts according to the course of flow of the exocrine secretions.

The *first* portion consists of the pancreatic tissue which lies in the gastrosplenic ligament and along the splenic and right gastroepiploic blood vessels and is drained by the large pancreatic duct which enters the bile duct about 1 cm. from the duodenum. The *second* portion lies in the mesoduodenum posterior to the bile duct and is drained by a large pancreatic duct that enters the bile duct less than 2 mm. from the duodenum. The *third* portion consists of many separate small lobules located along the full length of the bile duct; each lobule has its own duct entering the bile duct. Some of the ducts enter the bile duct at the junction of the latter with its tributaries from the different lobes of the liver.

By taking advantage of the fact that the lobules in each of the first two portions are connected by the pancreatic ducts, it is possible to remove each portion in one piece without fragmentation. The third portion must be removed lobule by lobule.

This is done with the aid of the dissecting microscope (10× magnification), which is used throughout the operation, and fine-tipped forceps. Another important anatomical

[2] This finely ground casein can be obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

fact is that the pancreatic tissue in the mesoduodenum lies on the right side of the inferior pancreaticoduodenal vessels. Hence, with the duodenum pulled to the extreme left side of the rat this pancreatic tissue can be removed without disturbing the blood flow in these vessels.

Surgical Procedure: After a fast of 8–18 hours the rat is anesthetized with ether and a mid-line abdominal incision is made. The stomach and spleen are pulled out of the abdominal cavity and placed on the chest wall. The transverse colon and its mesocolon are gently pulled caudad to expose the origin of the splenic vessels. With fine-tipped forceps the pancreatic tissue overlying the junction of the portal and splenic veins and along the first few millimeters of the splenic vessels is gently lifted cephalad without separating it from the rest of the gland.

With the rat rotated so that its head points to 7 o'clock, the spleen is taken between the left thumb and index finger with the finger tip placed under the splenic vessels. Starting in the abdomen and working toward the spleen with swabs (cotton rolled on tips of round tooth picks), the pancreas is pushed cephalad off the splenic vessels and off the vessels in the hilum of the spleen. The gastrosplenic ligament is separated from the spleen and is lifted onto the stomach. Any fragment of pancreatic tissue separated from the larger mass is immediately removed with the fine-tipped forceps. The spleen is then returned to the abdominal cavity.

The rat is rotated so that the head points to 12 o'clock and the gastrosplenic ligament is taken between the left index finger and thumb. Starting at the cardiac end, the gastrosplenic ligament is separated from the stomach. When pancreatic tissue is encountered it is pushed away from the stomach and blood vessels toward the tissue mass in the left hand. When only a band of pancreatic tissue is left across the front of the pylorus, the ligament is pulled out and the band is placed on the left index finger. The tissue on this finger is rubbed away from the abdomen with swabs to separate pancreatic tissue from the enclosed, large pancreatic duct. After exposing the duct, it is cut with scissors. Later, the remaining segment of this duct will be cut with the electrocautery close to the bile duct. This completes the removal of the first portion of the pancreas.

The duodenum is pulled out of the abdomen and to the right side of the rat. The colon is gently pulled to the opposite side in order to separate the mesoduodenum and mesocolon so as to expose the portal vein. The rat is now rotated so that the head points to 2 o'clock. The duodenum, taken between the left thumb and index finger, is pulled to the right side of the rat and the finger tip is placed under the bile duct. With cotton swabs the pancreas is pushed away from the bile duct toward the abdomen, starting at the duodenum and working toward the liver. About 0.2 cm. from the duodenum the large pancreatic duct from the second portion of pancreas is found and is cut near the bile duct with a small electrocautery. As the little lobules of the pancreas in the third portion are isolated, their ducts are cut either with the electrocautery or by pulling them apart between two pairs of forceps. About halfway between the duodenum and the liver the pancreatic duct from the first portion of pancreas will be found entering the bile duct. At this time the pancreatic tissue which lies along the course of the gastroduodenal vessels is separated from these vessels, duodenum and pylorus. The above pancreatic duct lies within this small mass of tissue. The dissection is continued along the blood vessels until the bile duct is encountered, at which time the pancreatic duct is cut next to the bile duct with the electrocautery.

The animal is rotated so that the head points to 8 o'clock. The duodenum is taken with the left index finger and thumb and pulled to the left side of the animal; the finger tip is placed under the bile duct. The remaining pancreatic tissue between the bile duct and the duodenum, as well as that along the bile duct, is now removed. Near the liver the pancreas is difficult to see if the bile duct is not pulled out a little. After cleaning the bile duct as much as possible, the removal of the third portion is completed.

After rotating the rat so that the head points to 9 o'clock, the duodenum, taken be-

tween the left thumb and index finger, is pulled to the left side of the rat and the finger tip is placed under the inferior pancreaticoduodenal vessels. The peripheral edge of the pancreatic tissue along the back of the abdomen is first lifted with the fine-tipped forceps and then with swabs, this pancreatic tissue and that along the inferior pancreaticoduodenal vessels are pushed toward the duodenum. After cleaning halfway to the duodenum, the other half of these vessels is cleaned by working from the opposite direction. The pancreatic tissue freed from these vessels is lifted caudad. Starting at

TABLE 2. SCHEDULE OF POSTOPERATIVE CARE FOR PANCREATECTOMIZED RATS

Postoperative day		Regular insulin, units[a]	Food-pancreatin suspension, ml.[b]	Penicillin-saline solution[c]
0	P.M.	0	0	yes
1	A.M.	0.5	0	yes
	P.M.	2.4	2[d]	no
2	A.M.	4	3	no
	P.M.	5	4	no
3	A.M.	6	5	no
	P.M.	9	7	no
After 3rd day				
	A.M.	9	7	no
	P.M.	9	7	no

[a] Insulin is always injected 1–2 hours before the morning meal and less than ½ hour before the afternoon meal. The second meal each day is given 6 or more hours after the first meal. The only type of insulin used in this study was regular insulin, which was kindly supplied by Laboratorios Armour of Buenos Aires, Argentina.

[b] This suspension contains 0.5 gm. of diet #4370 (Table 1) and 5 mg. of pancreatin (4 USP "Viokase," Viobin Corp., Monticello, Illinois) in each ml. It is prepared just before using by mixing 1 gm. of diet #4370, 10 mg. of pancreatin, and 1.3 ml. of water. The food suspension is fed through a #PE-90 polyethylene tubing which is attached to a 10 ml. BD Sana-Lok Control Syringe with a plastic tubing adapter (A-2625-B). (The tubing and adapter were purchased from Clay-Adams Co., New York, and the syringe from Becton, Dickinson and Co., Rutherford, New Jersey.)

[c] Subcutaneous injection of 5 ml. of 0.85% NaCl solution and 8,000 units of crystalline penicillin G.

[d] Drinking water is not given until after this meal.

the junction of these vessels with the duodenum, the pancreatic tissue lying along the duodenum is carefully pushed toward the mesentery and then, when freed, the removal of the second portion of the pancreas is completed.

The duodenum is then pulled to the right side of the rat and the portal vein is carefully inspected for pancreatic fragments, especially near its junction with duodenal and splenic vessels. The abdominal wall is closed with interrupted cotton sutures and the skin with a continuous cotton suture.

The operation takes about 30 minutes.

Postoperative Care: During the initial stages of this study many rats died of diabetic coma within the first few days after pancreatectomy because the severity of the diabetes was not adequately appreciated. As the dose of insulin was increased more animals survived but they developed large subcutaneous abscesses and had poor wound healing. When given the postoperative care outlined in Table 2 less than 5% of the rats died in diabetic coma, practically none had abscesses or poor wound healing, and the daily glycosuria was less than 0.2 gm. The treated rats did not have ketonuria, fatty livers, and fatty kidneys (see below).

Occasionally there may be a hypoglycemic reaction after the first insulin injection (0.5 units) if the blood glucose level is low; this is treated by tube-feeding 2 ml. of 15%

glucose solution. Because of the large amount of insulin needed the pancreatectomized rats are never given food *ad libitum*. With this regimen the average initial body weight loss of 5 gm. after the operation is regained by the fifth day and thereafter there is a continuous small weight gain.

OBSERVATIONS[3]

Immediate Effects of Pancreatectomy: At 19 hours after pancreatectomy the operated rats had hyperglycemia, glycosuria, ketonuria, and large amounts of fat in the liver and the renal tubules (intracellular). The average blood glucose level in 39 rats at this time was 410 (225–750) mg./100 ml.; as early as 5 hours after operation, it exceeded 150. If not treated with insulin, the operated rats died of diabetic coma within 48 hours after pancreatectomy (Table 3).

TABLE 3. EFFECT OF PANCREATECTOMY IN FASTING RATS[a]

Operation	No. of rats	Survival days	Body weight, gm.		Blood glucose, mg./100 ml., postoperative days[b]		Daily urinary excretion mg./100 gm. body weight[b]		
			Initial	Loss[b]	1st	2nd	Glucose	Nitrogen	Ketone bodies
Pancreatectomy	6	2	129	7 ± 1	367 ± 43	181 ± 55	47 ± 7	43 ± 4	30 ± 10
Laparatomy	7	7	118	8 ± 1	54 ± 5	43 ± 3	<10	57 ± 11	5 ± 3

[a] Although fasting was begun 17 hours before the operation, the data presented here were collected during the first two days after surgery. Less than 0.5% of the pancreas was left in the pancreatectomized rats; they were not treated with insulin.
[b] Mean ± standard error.

In addition to weakness, hypothermia, prostration, and hyperventilation, the rat in diabetic coma has a markedly swollen abdomen. The latter is the result of a great retention of fluid in the stomach, which occurs even when the rat is not given water to drink. In most cases aspiration of the fluid with a tube is not necessary because the stomach will empty within a few hours after injecting insulin if the coma subsides. Because of this relationship between the stomach and insulin in the diabetic rat insulin is given in the morning one to two hours before feeding (Table 2).

When treated with large amounts of insulin, as outlined in Table 2, the mortality due to diabetes was less than 5%. About 2% of the operated rats died within the first few days of hemorrhage or biliary peritonitis. Although 40% had obstruction of the bile duct, they survived 2 to 3 weeks. (For certain kinds of acute experiments these animals can be used without any serious difficulty.) The first sign of obstruction, bile in the urine, appeared 2 to 7 days postoperatively. If the biliuria lasted more than 2 days, the obstruction was usually permanent.

Effect of Small Remnants of Pancreas on the Need for Insulin and Pan-

[3] Unless otherwise stated, the average amount of pancreas left in the pancreatectomized rats described here was less than 0.5% of the gland.

creatin: When the survivors of the first 170 pancreatectomized rats were autopsied 4 to 5 months after the operation, about 20 mg. of pancreatic tissue, equivalent to 5% of the whole gland,[4] were found in each rat along the bile duct. Histological study[5] demonstrated that the acinar cells were normal and that the islets of Langerhans were small and few in number. The proportion of islets to acinar tissue was greatly subnormal. Beta cells were seen but no alpha cells. These rats, when fed 7 gm. of food per day, needed only 12 units of insulin to maintain glycosuria at less than 0.1 gm./day. Since their absorption of dietary nitrogen and the appearance of their feces were normal, no pancreatin was given. The average blood glucose level in these rats 19 hours after surgery was 310 mg./100 ml.

After modifying the method of cleaning the bile duct so as to remove more of the pancreas, the average amount of pancreas left was about 2 mg. (0.5% of the gland). Consequently, these pancreatectomized rats not only had a higher blood glucose level after surgery (410 mg./100 ml. at 19 hours) and greater insulin requirements, but also marked impairment of the absorption of food. Their feces were bulky, loose, foul smelling, fatty, and contained 59% of the dietary nitrogen. When pancreatin was given, 10 mg./gm. of dry diet, the feces immediately appeared normal and contained only 17% of the dietary nitrogen (normal under these experimental conditions).

Effect of Insulin on Glycosuria: About one month after pancreatectomy 8 rats which had pancreatic remnants of about 20 mg. at autopsy were used to study the effect of decreasing the dosage of insulin in rats tube-fed 7 gm. of food daily. When the dosage was decreased from 12 to 6 units the glycosuria increased five times (Table 4). When no insulin was given for only one day the average glycosuria was equivalent to 60% of the carbohydrate fed; three of the rats excreted an average of 57 mg. of ketone bodies and one rat died in diabetic coma. Although the ketonuria disappeared at once when the insulin dosage was increased to 12 units, the glycosuria decreased at a slow rate.

Blood Glucose Level After Feeding: Figure 1 presents the levels of blood glucose in pancreatectomized rats given insulin and food according to the schedule in Table 2. At the time food was given (one hour after insulin injection) the blood glucose was less than 100 mg./100 ml. and did not rise above this level until 3 hours later. At $7\frac{1}{2}$ hours after insulin injection the blood glucose was nearly at the initial level. In four rats given 7 units the blood glucose level at $7\frac{1}{2}$ hours was significantly higher than in those given 9 units.

If not given insulin when fed, the pancreatectomized rats had blood

[4] The ratio of pancreas to body weight in 8 female rats (of the Instituto de Biologia y Medicina Experimental), weighing 104 to 160 gm., was 292 mg./100 gm. (standard error = 18).

[5] The author is indebted to Dr. Adolfo F. Cardeza for the histological study.

TABLE 4. EFFECT OF INSULIN ON GLYCOSURIA AND KETONURIA IN 8 PANCREATECTOMIZED RATS TUBE-FED 7 GM. OF FOOD DAILY[a]

Regular Insulin, units/ days	Number of days	Daily urinary excretion[b]		Remarks
		Glucose, gm./100 gm. body weight	Ketone bodies, mg./100 gm. body weight	
12	6	0.04 ± 0.02	0	
6	4	0.23 ± 0.04	0	
0	1	2.07 ± 0.13	57 ± 14 (3 rats) 0 (5 rats)	1 died in diabetic coma
12	2	0.62 ± 0.14	0	
12	4	0.29 ± 0.07	0	

[a] An average of 20 mg. of pancreatic tissue (5% of the gland) was left in these animals.
[b] Mean ± standard error.

glucose levels between 800 and 1,500 mg./100 ml. the following morning and they died within 48 hours in diabetic coma.

Sensitivity to Small Amounts of Insulin: Although the amount of insulin needed by the pancreatectomized rat is large when food is given, very small amounts (3.3 milliunits) will significantly lower the fasting blood

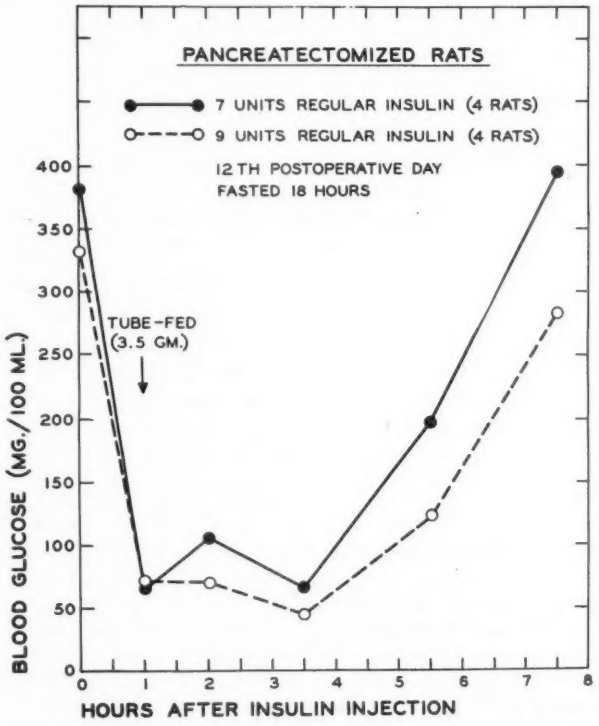

FIG. 1. Blood glucose curve after insulin and food in pancreatectomized rats.

glucose level. By measuring the drop in the fasting blood glucose during the first hour after injecting insulin subcutaneously, it was possible to clearly observe differences in effect between 3.3 and 10 and between 10 and 33 milliunits.[6] Whether greater sensitivity could be obtained by injecting the insulin by some other route, such as intravenously, was not studied.

DISCUSSION

These findings clearly demonstrate that if sufficient pancreas is removed the rat immediately develops very severe diabetes and has marked impairment of the absorption of food.[7] When 5% of the gland is left in the animal the diabetes is less severe and intestinal absorption is normal, similar to observations in the dog (3). Although the pancreatectomized rat survives a shorter time and excretes more ketone bodies during fasting than does the cat or dog, it excretes less glucose and nitrogen (Table 5).

TABLE 5. COMPARISON OF RAT, CAT, AND DOG: SURVIVAL AND URINARY EXCRETION OF GLUCOSE, NITROGEN, AND KETONE BODIES DURING FASTING OF PANCREATECTOMIZED ANIMALS

Species, data source	Survival, days	Daily urinary excretion, gm/kg. body weight		
		Glucose	Nitrogen	Ketone bodies
Rat	2	0.5	0.4	0.3
Cat (2)	4	3.2	1.4	0.1
Dog (1, 16)	10	2.8	1.0	0.2

In addition to having the usual signs of diabetes, the pancreatectomized rat in coma has marked retention of fluid in the stomach and large amounts of fat in the cells of the renal tubules. Similar observations have been described in human beings (17). These phenomena cannot be attributed to acute surgical trauma because they have been observed 3 months after pancreatectomy when insulin treatment was suspended.

The blood glucose levels in pancreatectomized rats at various times after the morning injection of insulin and after feeding are presented in Figure 1. It is reasonable to expect that a similar pattern would be repeated in the afternoon. If so, the blood glucose level would be maintained at relatively low levels during the time the food was being absorbed. For at least 8 hours during the night the blood glucose level probably averages

[6] In 10 pancreatectomized rats, tested with all doses, the decrease in the fasting blood glucose level in one hour was 40 ± 6 mg./100 ml. (mean $\pm$ standard error) after the subcutaneous injection of 3.3 milliunits of regular insulin, 122 ± 9 after 10 milliunits, and 196 ± 9 after 33 milliunits. Insulin was injected in 0.1 to 0.25 ml. of 0.85% NaCl solution with pH 8.8.

[7] These findings have been confirmed in rats of three different strains: Sprague-Dawley, Osborne-Mendel, and black rats of the animal colony at the National Institutes of Health.

350 mg./100 ml. With this regimen, however, only small amounts (less than 0.1 gm.) of glucose are excreted in the urine daily. There has been no evidence of sclerotic changes in the kidneys of these animals, some of which lived for more than 4 months (18).

SUMMARY

A method for pancreatectomizing rats, leaving less than 0.5% of the gland, is described. At 19 hours after the operation the rats had hyperglycemia, glycosuria, ketonuria, gastric retention of fluid, and large amounts of fat in the liver and renal tubules (intracellular). If not treated with insulin, they died within 48 hours in diabetic coma.

The pancreatectomized rats needed large amounts of insulin when food was given (2.5 units/gm. of food). On the other hand, when the rats were fasted they were sensitive to very small amounts of insulin (3.3 milliunits lowered the blood glucose level 40 mg./100 ml. in one hour).

The intestinal absorption of food was markedly impaired when more than 99% of the pancreas was removed; it was normal when 5% of the gland was left.

Acknowledgment

The author is greatly indebted to Dr. Bernardo A. Houssay for inspiration and guidance and for the cordial hospitality in the Instituto de Biologia y Medicina Experimental. The excellent assistance of Dr. Edgar Wagner and Miss Elena Ronov is also gratefully acknowledged.

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THE ISOLATION OF TWO CORTICOSTEROIDS FROM CATTLE BILE[1]

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THE presence of metabolites of adrenal cortical steroids in bile has been demonstrated in studies utilizing steroids labeled with tritium (1) or with C^{14} (2–10). In the rodent, excretion of isotopic metabolites by the biliary route was predominant (1–5), while in man, only a small percentage of the administered radioactivity was excreted by this pathway (6–8) or detected in the feces (7–10).

In the present paper, the isolation and identification of tetrahydrocortisol (THF) and tetrahydrocortisone (THE) from cattle bile are described. These steroids, probably metabolites of adrenal cortical steroids in this species, were obtained from bile that had been treated with a culture fluid containing bacterial β-glucuronidase.

EXPERIMENTAL

Materials

1. *Bile.* Cattle gall bladders with ligatured ducts were obtained from the slaughter house. The pooled bile, initially slightly alkaline, was adjusted to pH 4.5 with concentrated HCl prior to extraction with butanol. The period from the time of slaughter to adjustment of the pH was about 4 hours.

2. *Bacterial β-glucuronidase.* A culture fluid containing this enzyme was prepared and assayed as described by Doyle *et al.* (11). This fluid was centrifuged at 4° C to remove cells, and the supernatant layer filtered to remove free menthol. The clear filtrate was frozen until needed. When required, the thawed enzyme preparation was adjusted to pH 6.8 with HCl and purified by extraction five times with 0.4 volume of ether. Versene was added to give a concentration of 1 mg. per ml. of the enzyme preparation.

3. Versene, Eastman Organic Chemical No. 6354, was dissolved in water (10% solution), brought to pH 6.8, and purified by extraction with chloroform.

4. *Silica gel.* Davison, 100–200 mesh, was washed twelve times with methanol, air dried overnight, and dried an additional 8 hours under a 300 watt lamp.

5. Solvents were purified by appropriate conventional procedures.

Isolation of THF and THE from Cattle Bile

In a preliminary study, butanol extracts were prepared from 200 ml. aliquots of pooled cattle bile; the butanol was evaporated and the residues

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[1] The material presented herein is taken, in part, from a thesis submitted to the Graduate School of St. Louis University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

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dissolved in water. The aqueous solutions were extracted with chloroform, incubated with β-glucuronidase, and extracted again with chloroform. Each of the chloroform extracts was washed with dilute HCl, water, $NaHCO_3$ solution, and again with water. The chloroform was evaporated, and the residue partitioned between 70% ethanol and petroleum ether. The alcohol phase was evaporated, and the resulting residue fractionated by the paper chromatographic technique of Zaffaroni and Burton (12). Although no substances resembling α-ketolic steroids were extractable with chloroform before treatment with the enzyme preparation, two compounds which reduced ammoniacal silver nitrate (13) and blue tetrazolium (BT) (14) were present on the chromatograms of the chloroform extracts obtained after this treatment. No reducing compounds were detected in control experiments for this pilot study and for the large scale isolation work in which β-glucuronidase was incubated with water, the mixture extracted, the extract distilled and the residue chromatographed on paper. The control experiments gave assurance that the enzyme preparation did not yield reducing substances.

Twenty-six liters of pooled cattle bile (pH 4.5) were extracted four times with 0.5 volume of butanol, and the butanol extract was placed in the ice box for about 12 hours. During this time, water and some solid material separated and were removed. The butanol was evaporated *in vacuo* at 40° C, the residue dissolved in water and diluted to 104 liters. This solution was adjusted to pH 6.8 with 10% NaOH and extracted four times with 0.25 volume of chloroform, the organic phase being discarded. Versene solution was added to give 1 mg. of versene per ml. of the aqueous solution. The latter was then incubated with β-glucuronidase for 48 hours at 37° C. Twenty-eight liters of the enzyme preparation (1344 units per ml.) were added in two equal portions at an interval of 24 hours to give calculated activities of 157 and 141 units per ml. of the incubation mixture after addition of enzyme for the first and second time, respectively. β-Glucuronidase activity of the incubation mixture was measured 5 hours and 24 hours after each addition of enzyme. The average activity after 5 hours was 22 units per ml., and after 24 hours the value was about 0.7 units per ml.

To determine whether bacterial growth occurred, 1 ml. of the incubation mixture was withdrawn at intervals throughout the period of incubation and pour plates were made using 20 ml. portions of trypticase soy agar (Baltimore Biological Laboratory). No growth was observed in any of the plates after incubation at 37° C. for 24 hours.

Following enzymatic hydrolysis, the incubation mixture (pH 6.8) was extracted four times with 0.2 volume of chloroform. The extract was washed twice with 0.05 volume of 0.1 N HCl, once with 0.05 volume of water, twice with 0.05 volume of 0.1 N $NaHCO_3$, and three times with 0.05 volume of water. The chloroform was evaporated *in vacuo* at 40° C, the residue dissolved in 2.6 liters of 70% ethanol and this solution extracted

four times with an equal volume of petroleum ether. The combined petroleum ether phase was extracted twice with 1.0 liter of 70% ethanol and the ethanol washings added to the original alcohol phase.

After evaporation of the 70% ethanol *in vacuo* at 40° C, the residue was chromatographed on paper in the toluene-propylene glycol (methanol diluted) system (12, 13). Twenty-seven paper strips 19 cm. in width were employed, and development was carried out for 22 days in a "chromatocab."[3] After development, a number of test strips 2 mm. in width were cut longitudinally from each chromatogram. One test strip from each chromatogram was examined in ultraviolet light while others were treated with ammoniacal silver nitrate, BT, and 15% H_3PO_4 (15). By means of the test strips, seven zones (I through VII, counting from the starting line) of which only III and V reduced BT were located on 22 of the 27 chromatograms; THF and THE were subsequently isolated from Zones III and V, respectively. Since the material on the remaining five chromatograms did not separate satisfactorily, it was discarded, and the amount of bile represented in the subsequent procedures was about 21 liters.

The remaining untreated portions of the chromatograms were cut into sections, corresponding to the seven zones. Zones III and V were cut into small pieces and the compounds eluted by washing the paper four times with ethanol. After filtration and evaporation of the ethanol, the residues were partitioned between chloroform and water to remove propylene glycol (16). The chloroform solutions were evaporated and each of the residues was again chromatographed on paper in the toluene-propylene glycol (methanol diluted) system. The chromatograms were developed for 30 days, the two reducing zones were eluted, the eluates were evaporated and the residues partitioned between chloroform and water. Both compounds were contaminated with a yellow pigment and possibly with other material. The further purification of each of these compounds will be discussed separately.

Purification and Identification of the Reducing Substance in Zone III (Compound III, THF)

The residue (wt. 36 mg.) from the second chromatography contained the reducing compound. It was chromatographed on 30 gm. of silica gel (17), the column being developed with mixtures of chloroform and ethanol. Compound III was eluted with 4% ethanol in chloroform. This was determined by depositing 0.1 ml. (1%) of each fraction on a separate strip of Whatman No. 1 filter paper, in as small an area as possible, and testing the spot with the BT reagent. 1.2 mg. of material was obtained by combining the fractions containing the reducing substance.

Since this product was not pure, it was partitioned between water and petroleum ether and between water and benzene. The reducing substance

[3] Chromatography Division, University Apparatus Company, Berkeley, California.

remained almost entirely in the water, from which it was extracted with chloroform. The residue obtained by evaporation of the chloroform was chromatographed on a paper strip 19 cm. in width, using the toluene:methanol:water (10:5:5) system (B4-system) described by Bush (18). Compound III was detected by cutting several test strips from the chromatogram and treating these with BT. The reducing substance was eluted from the remainder of the chromatogram by washing the portion of the paper containing it five times with methanol. After filtration and evaporation of the eluate, the residue was partitioned between chloroform and water. The residue obtained by evaporation of the chloroform contained approximately 0.3 mg. of material, based on assay by the method of Silber and Porter (19) and calculated as THF.

Since Compound III showed polarity greater than Compound V in paper chromatography and both compounds gave a positive Silber-Porter reaction, it was suspected that Compound III might be THF. Accordingly 2 mg. of authentic THF were purified by paper chromatography in Bush's B4-system. After crystallization from a mixture of acetone and petroleum ether, the melting point[4] of a small crystal was 201–203° C. Attempts to crystallize Compound III from acetone and petroleum ether were unsuccessful. However, the sulfuric acid spectrum of a small crystal of THF was identical with that of a small portion of amorphous Compound III (Figure 1). In addition, both products, as well as a mixture, moved 8 cm. whereas THE moved 11.5 cm. during development for 24 hours in Bush's B4-system of paper chromatography.

Compound III and THF were acetylated with pyridine and acetic anhydride at 25° C for 24 hours, and the acetylated residues were purified by paper chromatography in the petroleum ether:methanol:water (10:8:2) system (A-system) described by Bush (18). The reducing substances were located on a test strip of the chromatograms by the BT reaction. They were eluted from the remaining portions of the chromatograms by washing the sections of the paper containing them five times with a mixture containing equal volumes of chloroform and methanol. The eluates were filtered and evaporated and the residues partitioned between chloroform and water. After evaporation of the chloroform, the acetates were crystallized from aqueous methanol. The melting point of a small crystal of each substance was determined: THF-3,21-diacetate, 215–218° C; Compound III-acetate, 215–218° C. Lieberman *et al.* reported that large crystals of THF-3,21-diacetate melted at 212°, 218–222° while small crystals melted at 208–211° C (21).

The absorption spectra in sulfuric acid of small crystals of the two acetates were similar (Figure 1). Using the mother liquors from the crystalline products, each of the acetates as well as a mixture moved 6.6 cm.

[4] All melting points were determined on a Fisher-Johns apparatus and are recorded as read.

during development for 11 hours in Bush's A-system of paper chromatography; THE-diacetate moved 11.7 cm. under identical conditions.

The remaining crystals of Compound III-acetate, 0.055 mg., and the residue from the mother liquor, 0.060 mg., as measured by the Silber and Porter method, were combined and the infrared spectrum[5] of this sample was compared with the spectrum of 0.38 mg. of crystalline THF-acetate.

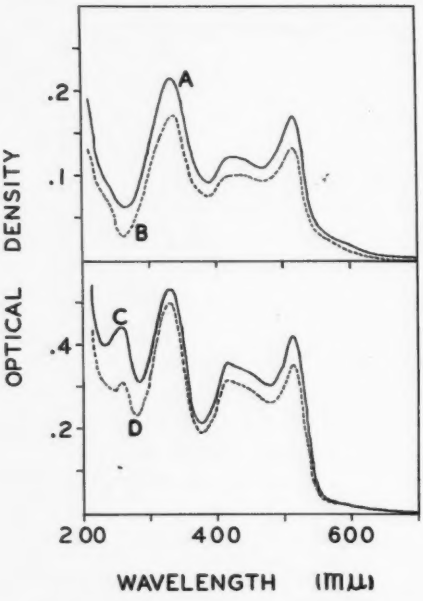

FIG. 1. Absorption spectra of compounds in sulfuric acid, according to the method of Zaffaroni (20). Curve A, THF; Curve B, Compound III from cattle bile; Curve C, THF-3,21-diacetate; Curve D, Compound III-acetate. Since a small portion of each substance was placed directly in a microcell and covered with sulfuric acid, the concentrations of the solutions were unknown.

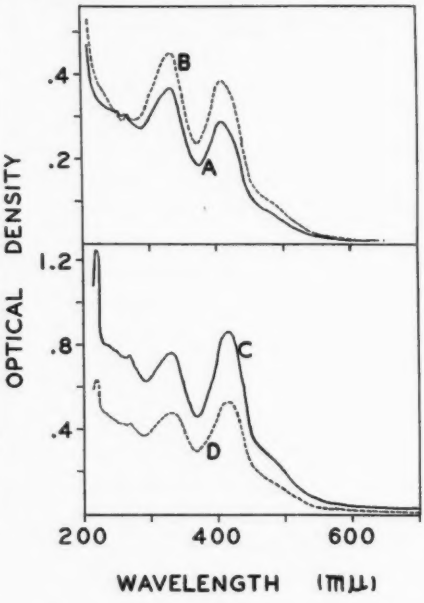

FIG. 2. Absorption spectra of compounds in sulfuric acid, according to the method of Zaffaroni (20). Curve A, THE; Curve B, Compound V from cattle bile; Curve C, THE-3,21-diacetate; Curve D, Compound V-acetate. Since a small portion of each substance was placed directly in a microcell and covered with sulfuric acid, the concentrations of the solutions were unknown.

While the two spectra were comparable, the fingerprint regions were not well defined. Several of the major bands for Compound III-acetate were as follows: ν_{max}^{KBr} 3610–3534 (broad), 1763 (inflection), 1745, and 1246 cm.$^{-1}$. The corresponding bands for THF-acetate were: ν_{max}^{KBr} 3597, 1757 (inflection), 1748, and 1246 cm.$^{-1}$.

[5] The author is indebted to Dr. Theodore E. Weichselbaum and Mr. Leon E. Ashford of the Washington University School of Medicine, St. Louis, Mo., for the determination of the infrared spectra.

Purification and Identification of the Reducing Substance in Zone V (Compound V, THE)

The eluate of the reducing substance in Zone V weighed 60 mg. after chromatography in the toluene-propylene glycol system for the second time. It was chromatographed on 26 gm. of silica gel. The reducing material was eluted with 4% ethanol in chloroform, and the fractions containing it were combined and evaporated. By assay with the Porter-Silber (22) procedure the residue, which weighed 6 mg., contained 2.2 mg. of reducing compound calculated as THE. The residue was chromatographed on 8 gm. of silica gel, and 3 mg. of material containing 1.3 mg. of "THE" was obtained.

After partitioning this residue between water and petroleum ether and between water and benzene, 1.3 mg. of material containing 0.9 mg. of "THE" was extracted from the aqueous phase with chloroform. Since attempts to crystallize the product were unsuccessful, it was chromatographed on paper in Bush's B4-system. After elution of the reducing substance from the chromatogram with five portions of methanol, the eluate was filtered and evaporated, and the residue partitioned between chloroform and water. The chloroform was evaporated, and Compound V was crystallized from aqueous methanol. Approximately 0.2 mg. of crystals were obtained, based on assay by the Porter and Silber method and calculated as THE. The melting point of the crystals was not determined.

The absorption spectra in sulfuric acid of small crystals of Compound V and THE[6] were similar (Figure 2). Moreover, both products, and a mixture of them, moved 11.5 cm. during development for 24 hours in Bush's B4-system of paper chromatography. THF moved 8 cm. under identical conditions.

Crystalline Compound V was combined with the residue obtained from the mother liquor following crystallization, and the mixture was acetylated with pyridine and acetic anhydride at 25° C for 24 hours. The acetylated product was purified by paper chromatography (Bush's A-system), the eluate evaporated and the residue partitioned between chloroform and water. The chloroform was evaporated and the residue crystallized from aqueous methanol. Authentic THE-3,21-diacetate[6] was purified by chromatography on silica gel and crystallized from aqueous methanol. A mixed melting point determination of crushed crystals of the two products gave the following results: THE-3,21-diacetate, 236–242°; Compound V-acetate, 232–235°; mixture, 235–239°. Other workers have reported melting points of 225–228° (16), 233–236° (23), 226–228° (24), and 234–238° C (25). The sulfuric acid spectra of small crystals of the two acetates were identical (Figure 2).

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[6] The THE and THE-3,21-diacetate used in this study were generously donated by Merck and Company, Inc., Rahway, New Jersey.

The weight of the remaining crystals of Compound V-acetate was 0.14 mg. The infrared spectrum[5] of this sample was comparable to that of 0.5 mg. of crystalline THE-3,21-diacetate, although definition in the fingerprint region was not well defined. Several of the major bands for Compound V-acetate were as follows: ν_{max}^{KBr} 3521, 1754 (inflection), 1745 (inflection), 1736, 1727 (inflection), and 1250 cm.$^{-1}$. The corresponding bands for THE-3,21-diacetate were: ν_{max}^{KBr} 3533, 1754 (inflection), 1745 (inflection), 1736, 1727, and 1250 cm.$^{-1}$.

After determination of the infrared spectra, Compound V-acetate and THE-3,21-diacetate were recovered from the KBr pellets. The two products, and a mixture of them, moved 11.5 cm. during development for 11 hours in Bush's A-system of paper chromatography.

DISCUSSION

The following steroids have been previously isolated from cattle bile by Pearlman and co-workers: pregnane-3α-ol-20-one, pregnane-3α,20β-diol, and etiocholane-3α,17β-diol (26); allopregnane-3β,20β-diol (27); and estrone (28). Estriol, probably of biliary origin, has been isolated from meconium (the intestinal contents of the fetus and newborn) by Kinsella, *et al.* (29). The presence of metabolites of adrenal cortical steroids in the bile of rodents (1–5) and of man (6–8) has been demonstrated in studies utilizing steroids labeled with radioactive isotopes, and in the present paper the isolation of THF and THE from cattle bile is reported.

Although the quantitative determination of THF and THE in cattle bile was not attempted, the recovery of steroids was determined for two of the purification methods used in the isolation work: 1) 0.8 mg. quantities of crystalline THE were chromatographed on paper strips 19 cm. in width, utilizing the toluene-propylene glycol system. After development for 23 days, assay by the Silber and Porter method of the material eluted from the chromatograms indicated approximately complete recovery of the steroid; 2) 0.4 mg. quantities of crystalline THF were chromatographed on 3 gm. and 24 gm. of silica gel. The columns were developed with mixtures of chloroform and ethanol and recoveries of the steroid, based on Silber and Porter assay, were 84 and 12%, respectively. The recoveries of 1.7 mg. quantities of crystalline THE, similarly chromatographed, were 92 and 44%, respectively. Moreover, on the basis of the Porter and Silber assay, only 60% of Compound V was recovered following chromatography on 8 gm. of silica gel in one instance in the purification of corticoids of bile.

SUMMARY

Tetrahydrocortisol and tetrahydrocortisone have been isolated from cattle bile after preliminary treatment of the bile with β-glucuronidase. Although less than 1 mg. of each of these steroids was obtained from 21 liters of bile, an examination of the purification procedures indicated that considerably larger quantities escaped isolation.

THF from cattle bile was characterized by its sulfuric acid spectrum and by a mixed paper chromatogram. The acetate was prepared and identified by a mixed paper chromatogram, melting point, and sulfuric acid and infrared spectra.

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THE METABOLISM OF MUCOPOLYSACCHARIDES IN ANIMALS: THE EFFECT OF CORTISONE AND HYDROCORTISONE ON RAT SKIN[1]

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THE administration of cortisone is reported to delay the formation of connective tissue in wounds of rabbits (1) while prolonged percutaneous application of cortisone and hydrocortisone to rats causes regressive changes in the dermal connective tissue (2). Layton (3) found that sulfate fixation in the skin of intact rats is inhibited by cortisone administration. Autoradiographs show a considerably smaller S^{35} content in the skin of the cortisone-treated rat than of the control animal (4).

The results of Layton (3) and of Boström and Odeblad (4), which are based on the use of S^{35}, imply that the formation of sulfated mucopolysaccharides is inhibited in the skin of cortisone-treated animals. While such data might be related to chondroitinsulfuric acid (CSA) synthesis, the latter investigators (4) claimed a retarding effect of cortisone only on the exchange of the ester sulfate group of the CSA molecule. In a recent publication cortisone is reported to inhibit the incorporation of sulfate and thereby increase the relative concentration of nonsulfated mucopolysaccharides in syphilomas of rabbits (5). This conclusion is based solely on the weights of the mucopolysaccharides isolated by a technique which yields questionable recoveries.

Boström and Odeblad (4) demonstrated an inhibition of S^{35} uptake by the CSA isolated from cartilage slices after incubation with cortisone. The *in vitro* response of the cartilage slice to cortisone and cortisone acetate was confirmed by Clark and Umbreit (6). However, they found that the addition of hydrocortisone or its acetate ester to the *in vitro* system enhanced the uptake of S^{35} by cartilage and they concluded that the *in vitro* response of cartilage to the adrenal steroids appeared to be unrelated to the physiological action of these substances. The report that cortisone acetate has no effect while free cortisone increases the *in vitro* uptake of S^{35} by granulation tissue of guinea pigs (7) corroborates such a view.

As part of an investigation concerned with the influence of the endocrine glands on the connective tissues of animals, the present study was under-

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taken to determine the effect of administered cortisone and hydrocortisone on the synthesis of hyaluronic acid (HA) and CSA in skin, as measured by the radioactivity of the isolated mucopolysaccharides. Following the administration of C^{14}-carboxyl-labeled acetate and $Na_2S^{35}O_4$, it was possible to demonstrate a decrease in the uptake of C^{14} by HA and in the uptake of C^{14} and S^{35} by CSA in animals pretreated with cortisone. When hydrocortisone was injected subsequent to labeling of the connective tissue mucopolysaccharides, the rate of HA and CSA turnover gradually decreased with time.

MATERIALS AND METHODS

Intact male adult rats of the Sprague-Dawley strain, weighing between 180 and 220 gm., were used. The animals were maintained on Rockland chow and were allowed food and water *ad libitum* throughout the experimental period.

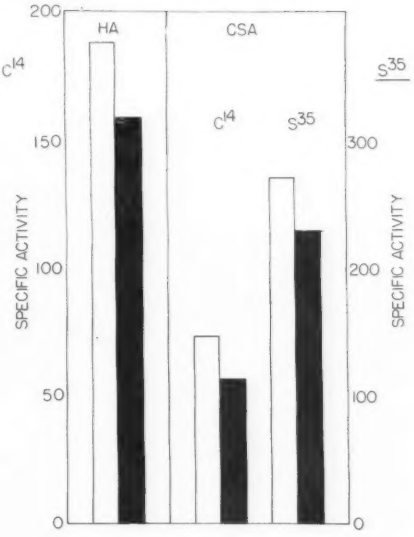

FIG. 1. Open bars represent the radioactivity of the hyaluronic acid (HA) and chondroitinsulfuric acid (CSA) isolated from the skin of normal rats; solid bars, that of cortisone acetate-treated rats. The specific activity was determined as counts per mg. of $BaCO_3$ and $BaSO_4$ for the C^{14} and S^{35}, respectively, 24 hours after the administration of C^{14}-labeled acetate and $Na_2S^{35}O_4$.

Suspensions of crystalline cortisone acetate[2] and hydrocortisone acetate[3] were prepared daily for injection by grinding the required amount of steroid in a mortar with saline and a drop of Tween 80.[4] The mixture was diluted to a final concentration of 10 mg. of steroid per ml. of saline, each 15 ml. of which contained 2 drops of Tween 80.

The HA and CSA were isolated from the skin by a method previously described (8).

The separated mucopolysaccharides were oxidized to CO_2 and the C^{14} counted as $BaCO_3$ at infinite thinness. Silver wire was used in the combustion tube filling to assure the complete removal of the S^{35} during the oxidation of the CSA. Appropriate control experiments showed no contamination of C^{14} samples with S^{35}.

[2] Crystalline cortisone acetate was generously supplied by Merck and Co.

[3] Crystalline hydrocortisone acetate was a gift from the Upjohn Co.

[4] Tween 80 (polyoxyethylene sorbitan mono-oleate) was supplied by the Atlas Powder Co.

The sulfate of the CSA was precipitated as $BaSO_4$ after hydrolysis of the mucopolysaccharide in 4 *N* HCl for 8 hours in boiling water. The S^{35} was counted as the $BaSO_4$ at infinite thinness.

EXPERIMENTAL METHODS AND RESULTS

The Effect of Cortisone Acetate on Uptake C^{14} and S^{35}. A group of 10 rats was injected subcutaneously each day with 5 mg. of cortisone acetate. On the fourth day each rat

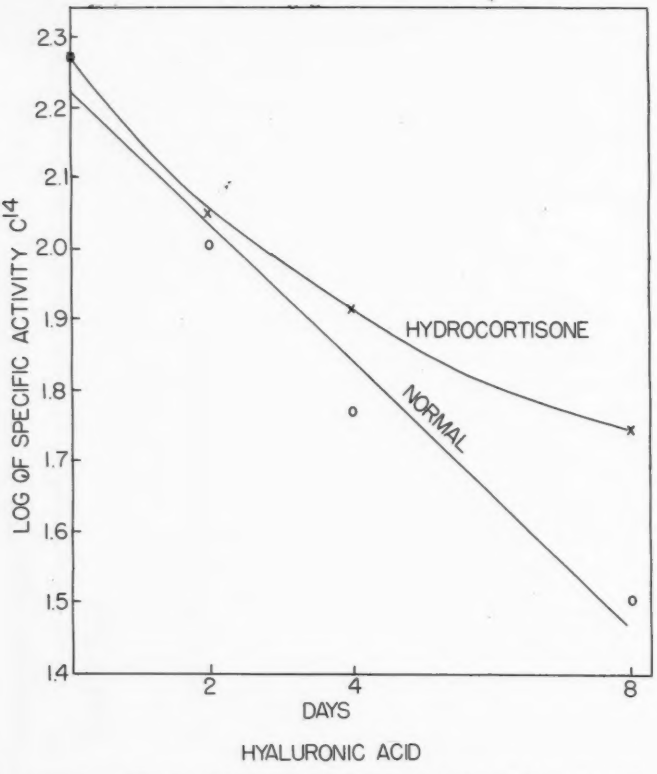

FIG. 2. A semilogarithmic plot of the C^{14} of the HA isolated from the skin of normal rats (o) and of rats treated with hydrocortisone acetate (x). The decay curve of the HA from the normal animals was obtained by the method of least squares. The curve of the HA from the animals treated with hydrocortisone acetate was drawn through the experimentally determined points. Injections of hydrocortisone acetate were started at zero time as described in the text.

received, in addition, a single subcutaneous injection of 62.5 μc. of C^{14}-carboxyl-labeled acetate and 30 μc. of $Na_2S^{35}O_4$ as an isotonic mixture. A group of 10 untreated rats was injected similarly with the radioactive substances. The 2 groups of animals were sacrificed 24 hours later and the HA and CSA were isolated from the pooled rat skins of the respective groups.

After 4 days of cortisone acetate administration, the uptake of C^{14} by the HA and CSA isolated from skin was 85 and 77%, respectively, of that

in the control rats. The S^{35} uptake by the CSA was also decreased (Fig. 1) in the cortisone-treated animals. This effect of cortisone on the uptake of S^{35} by the CSA of the skin is similar to the decreased incorporation of S^{35} in the CSA isolated from the cartilage of cortisone-treated rats (4).

The Effect of Hydrocortisone Acetate on Turnover. Seventy rats were injected subcutaneously with 62.5 μc. of C^{14}-carboxyl-labeled acetate and 30 μc. of $Na_2S^{35}O_4$ as an isotonic mixture. One group of 10 rats was sacrificed 24 hours later. The radioactivity

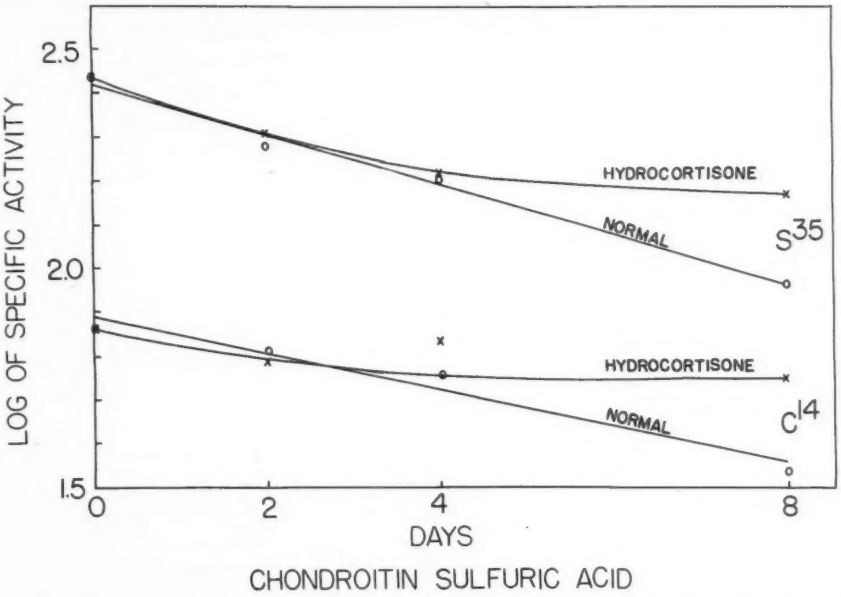

FIG. 3. A semilogarithmic plot of the C^{14} and S^{35} of the CSA isolated from the skin of normal rats (o) and of rats treated with hydrocortisone acetate (x). The decay curves for CSA from the normal rats were obtained by the method of least squares. The curves of the CSA from the animals treated with hydrocortisone acetate were drawn through the experimentally determined points. Injections of hydrocortisone acetate were started at zero time as described in the text.

of the HA and CSA isolated from the pool of 10 rat skins represents the uptake by untreated animals at zero time. At this time daily subcutaneous injections of 5 mg. of hydrocortisone acetate per animal were started. Three groups of 10 rats received the adrenal steroid and the remaining 3 groups of 10 rats served as control animals. One group each of treated and untreated animals were then killed 3, 5, and 9 days, respectively, after the administration of the radioactive mixture and the HA and CSA were isolated from each group comprising a pool of 10 rat skins.

The apparent half-life times in the skin of the normal rat, as calculated from the lines of best fit, are 3.1 days for the HA (Fig. 2) and 7.2 and 5.3 days for the CSA on the basis of the C^{14} and S^{35} decay rates, respectively (Fig. 3). These values are in agreement with those previously published (9).

When treatment with hydrocortisone acetate is initiated at the time of maximal labeling, it is possible to demonstrate a gradual decrease in the rate of turnover of the mucopolysaccharides in the skin (Fig. 2 and 3). The inhibitory effect of the steroid is manifest after 4 days of treatment and becomes more pronounced by the end of the experimental period.

DISCUSSION

The results of this study confirm and extend earlier findings (3, 4) regarding an inhibition by cortisone of S^{35} uptake in the CSA of skin. Cortisone and hydrocortisone appear to depress not only the incorporation of sulfate in CSA, but also the incorporation of the N-acetyl moiety in both the HA and the CSA of rat skin. That the radioactivity of the two mucopolysaccharides isolated from the skin of animals injected with C^{14}-carboxyl-labeled acetate is confined to the N-acetyl component has been demonstrated in a previous publication (10). While it is true that in the hormone-treated animal the radioactivities measured demonstrate a decreased turnover of the N-acetyl and ester sulfate groups, these measurements reflect, in fact, decreased synthesis of the entire polysaccharide molecule (9). A defect in glucose metabolism due to excessive adrenal cortical hormones may well interfere with the utilization of glucose as a precursor of the hexosamine and hexuronic acid moieties (9) and thus depress mucopolysaccharide formation.

The exact mechanism of action of the adrenal steroids on mucopolysaccharide synthesis cannot be deduced from the data obtained in these experiments. It is significant, however, that a decrease in the rate of turnover of the mucopolysaccharides in skin is found not only in the intact rat treated with adrenal steroids but also in the insulin-deficient animal (11).

SUMMARY

The uptake of C^{14} by the hyaluronic acid (HA) and chondroitinsulfuric acid (CSA) and the uptake of S^{35} by the CSA was inhibited in the skin of rats injected with cortisone acetate.

Daily injections of hydrocortisone acetate resulted in a gradual decrease in the rate of turnover of the HA and CSA in skin.

Acknowledgment

The authors are indebted to Kathryn F. Dewey for technical assistance.

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THE *IN VITRO* HYDROXYLATION OF 21-CARBON STEROIDS BY RAT ADRENAL GLANDS[1]

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MARKED species variations in the relative proportions of 17α-hydroxycorticosterone (cortisol) and corticosterone in mammalian adrenocortical secretions have been reported (1, 2). Some animals, like the monkey, appear to secrete primarily cortisol, while others, like the rabbit, primarily corticosterone. A third group of animals, of which the dog is representative, secretes a mixture of the two hormones, with cortisol usually occurring in somewhat greater quantities.

In the case of the laboratory rat, most investigators have found that this species secretes corticosterone, aldosterone and a varying number of unidentified substances but no readily detectable quantities of cortisol (1, 3, 4, 13).

The present study was undertaken in an attempt to discover the reason for the impaired ability of the rat to hydroxylate cortical steroids at carbon 17. In this regard, it was felt that observations relating to the capacity of the rat adrenal cortex to hydroxylate a variety of 21-carbon steroids under *in vitro* conditions might prove informative. The following experiments were accordingly performed.

MATERIALS AND METHODS

Quartered adrenal glands, obtained from female rats of the Sherman strain weighing about 150 gm., were incubated in an artificial medium for two and one-half hours at 37° C in an atmosphere of oxygen. Routinely, twenty rats were employed in each experiment, each incubation flask receiving the glands from two animals. Steroid substrates were added to five of the ten flasks, while the remainder received no steroids and served as controls. The steroids added to the flasks were dissolved in 95% ethanol and their concentrations in this solvent so adjusted that the requisite amount of substrate was contained in a volume of 0.1 ml. Each of the control flasks received 0.1 ml. of the solvent.

Ethyl acetate extracts of the contents of the incubation flasks were chromatographed on silica gel columns, an appropriate solvent system being used to obtain a fraction containing the adrenocortical steroids. The steroids obtained from each group of five flasks were merged into one combined extract; aliquots from this extract were taken for the determination of formaldehydogenic substances (FS) and Porter-Silber chromo-

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[2] John and Mary R. Markle Scholar in Medical Science.

gens (PSC), the latter procedure being performed as described by the authors (5). The details of the procedures described to this point have been published previously (3). The remainder of the extract was reserved for examination by paper chromatographic methods.

In the case of the flasks to which steroids had been added, from their content of FS and PSC was subtracted the amounts of like substances found in the control flasks. The differences in steroid content were then presumed to be a measure of the extent to which the substrates had been hydroxylated at carbons 17 and 21 or only at carbon 21. (Steroids with an α-ketolic or dihydroxyacetone-type side chain would be formaldehydogenic but only those having the latter type of side chain would be measured in the Porter-Silber procedure.) To compute the extent of the conversions of the substrates to more polar compounds in terms of micromoles, the following assumptions were made: 1) the molecular weight of the PSC was 362 or approximately that of cortisol; and 2) the molecular weight of the FS could be estimated by assuming them to represent essentially a mixture of corticosterone and cortisol, the proportion of each in the mixture being indicated by the extent to which the formaldehydogenic substances were also Porter-Silber chromogens.

For purposes of paper chromatography, the extracts were put on 42 cm.-long strips of Whatman No. 1 paper. Corticosterone, cortisol, cortisone and 11-desoxycorticosterone were employed routinely as reference standards on one limb of the chromatogram. The chromatograms were first placed in a hexane tank and overrun with this solvent for 18 to 48 hours. This removed a large part of the ethyl acetate and methanol-soluble yellowish material which occurred in the extracts and which could not be eliminated by prior chromatography on silica gel columns. Following the hexane treatment, the chloroform-formamide system of Zaffaroni (6) was employed to separate the individual steroids. The reactive areas on the paper chromatograms were detected by their absorption at 240 mμ and their effect on blue tetrazolium.

RESULTS

Steroidogenesis from endogenous substrates

The content of formaldehydogenic substances (FS) and Porter-Silber chromogens (PSC) in the control flasks both before and after the incubation period are reported in Table 1. The rate of formation of FS and PSC

TABLE 1. *In vitro* ADRENOCORTICAL STEROID FORMATION FROM ENDOGENOUS SUBSTRATES

Treatment	No. rats	No.[4] det'ns.	Formaldehydogenic substances (FS)		Porter-Silber chromogens (PSC)		
			μg.[1] / 100 mg. adrenals	Net output[2] μg. / 100 mg. adrenals	μg.[1] / 100 mg. adrenals	Net output,[2] μg. / 100 mg. adrenals	PSC / FS
Unincubated	38	2	13($\pm$2.5)[3]	—	<1	—	—
Incubated	100	9	47($\pm$3.0)	34	9($\pm$0.6)	9	0.26

[1] Average values expressed as weight equivalents of 11-desoxycorticosterone.
[2] Per two and one-half hours.
[3] Standard error of the mean.
[4] Number of merged extracts that were analyzed. Each merged extract represented the steroids produced by the adrenals of 10–20 rats.

from endogenous substrates in the two and one-half hours of incubation can thus be measured and is so indicated in the table.

The data reveal that a preponderance of the substances formed (74%) are formaldehydogenic but do not possess the structural configuration required for reaction with the Porter-Silver reagent. The amount of PSC in the rat adrenal prior to incubation is negligible and does not increase greatly in the incubation period (9 μg./100 mg. adrenals/2.5 hrs.). More appreciable quantities of FS are formed (34 μg./100 mg. adrenals/2.5 hrs.).

TABLE 2. THE *in vitro* HYDROXYLATION OF VARIOUS 21-CARBON STEROIDS BY RAT ADRENAL GLANDS

Steroid supplied	Final conc. molar	No. rats	Av. wt. of adrenals, mg. / flask	No. det'ns.	Amount supplied, μmol.×10² / 100 mg.	Formaldehydogenic substances: Amount converted, μmol.×10² / 100 mg.	Formaldehydogenic substances: % converted	Porter-Silber chromogens: Amount converted, μmol.×10² / 100 mg.	Porter-Silber chromogens: % converted	PSC / FS
Δ^5-Pregen-3β-ol-20-one	10^{-4}	50	77.4	4	37.8	6.6[1] (4.8–8.6)[2]	17.5	0.5 (0.1–2.5)	1.3	0.08
Progesterone	10^{-4}	60	68.1	5	42.5	11.8 (7.1–16.6)	27.8	2.7 (1.3–3.5)	6.4	0.23
11β-Hydroxy-Progesterone	10^{-4}	60	70.8	5	42.8	17.3 (15.0–21.5)	40.4	0.6 (0.3–1.3)	1.4	0.03
17α-Hydroxy-Progesterone	10^{-4}	60	79.8	3	37.9	9.7 (8.8–10.3)	25.6	—	—	—
11-Desoxy-corticosterone	10^{-4}	60	80.4	4	37.7	—	—	3.9 (2.9–5.2)	10.4	—
Corticosterone	10^{-4}	60	88.0	4	34.5	—	—	1.1 (0.9–1.4)	3.1	—
Progesterone	10^{-3}	60	79.1	4	366.0	20.2 (18.8–20.8)	5.5	3.8 (2.8–4.4)	1.0	0.19
11-Desoxy-corticosterone	10^{-3}	60	91.9	3	329.0	—	—	9.6 (8.9–10.4)	2.9	—

[1] Mean value.
[2] Range of the values.

The data in Table 1 were employed, as explained above, as control values in determining the extent to which the added steroids were converted to more polar compounds.

The in vitro *hydroxylation of steroid substrates*

To determine if structural configuration would affect the extent to which 21-carbon steroids would be hydroxylated *in vitro* at carbons 17 and 21 by rat adrenal glands, a variety of compounds were added to the incubation flasks. The choice of the compounds used was influenced, in a large measure, by the scheme of corticosteroid biosynthesis proposed by Pincus (7). The results obtained are shown in Table 2.

It is of particular interest to note that, while 11β-hydroxyprogesterone was converted to FS more readily than its 11-desoxy congener, progesterone was converted to PSC four to five times as readily as was 11β-hydroxyprogesterone. A similar comparison between 11-desoxycorticosterone and corticosterone indicates that the former compound was converted to PSC three to four times more readily than the latter.

A comparison of the data obtained from the incubations containing progesterone and from those containing 17α-hydroxyprogesterone suggests that prior hydroxylation at carbon 17 does not significantly affect subsequent hydroxylation at carbon 21.

The capacity of the hydroxylating enzymes

One possible explanation for the failure of the rat adrenal cortex to synthesize large quantities of steroids hydroxylated at carbon 17 would be to postulate that only very limited amounts of a "17α-hydroxylase" were present in the gland. To test this possibility, incubations were prepared to which were added ten times the quantity of progesterone and of 11-desoxycorticosterone that were employed originally. The results are shown at the bottom of Table 2.

The quantities of steroids produced in these experiments suggest that the capacities of the hydroxylating enzymes were not exceeded when the substrate was supplied in a concentration of $10^{-4}M$ and that the rate of production of PSC could increase with a large increase in substrate concentration.

Tentative identification of the conversion products

Paper chromatographic analyses were employed to identify tentatively the compounds to which the steroid substrates had been converted during the incubation period. For this purpose, the steroids produced *in vitro* by groups of from ten to thirty rats were put on the chromatograms.

A. Control flasks

When the contents of the control flasks, which contained steroids synthesized solely from endogenous substrates, were examined, the greatest quantity of reactive material, by visual criteria, appeared to be in a zone having a mobility similar to that of corticosterone. By incubating the adrenals of 120 rats in the presence of 11-desoxycorticosterone, a sufficient amount of the material occurring in this zone was obtained so that it could be crystallized, acetylated and submitted to infra red spectroscopic analysis.[3] Absorption bands at 1737 and 1756 cm^{-1} indicated the 21-acetoxy-20-ketone configuration and one at 1678 cm^{-1}, the Δ^4-3-ketone. The presence of a free hydroxyl group was indicated by absorption at 3600 cm^{-1}. Possibly because the sample submitted for analysis could not be gotten completely free of the yellowish contaminant mentioned above, the spectrum in the "fingerprint" region was poorly resolved, being similar to but not identical with that of corticosterone acetate.

The chromatograms of the contents of the control flasks also contained a reactive zone having a mobility similar to that of aldosterone but not that of cortisone. The material in this zone absorbed ultra violet light at

[3] Kindly performed for the author in the laboratories of Dr. Seymour Lieberman.

240 mμ and reduced blue tetrazolium. Another zone seen constantly was one having a mobility similar to that of cortisol. The material in this zone absorbed ultra violet light but would not reduce blue tetrazolium, so it is to be doubted that it contained appreciable quantities of cortisol. Two additional zones which did not reduce blue tetrazolium were detected under ultra violet light. One was more polar than cortisol and one less polar than corticosterone.

B. Flasks supplied with steroid substrates

The contents of the flasks which received pregnenolone, 11β-hydroxyprogesterone and corticosterone contained no reactive zones not seen on the control chromatograms.

On the bases of mobility, ability to absorb ultra violet light and to react with blue tetrazolium, zones were detected on the paper chromatograms that suggested the conversion of progesterone to 11-desoxycorticosterone and 17α-hydroxyprogesterone to cortisol.

The contents of the flasks which received 11-desoxycorticosterone, when chromatographed on paper, revealed the presence of a reactive zone having a mobility intermediate between cortisone and corticosterone. The material in this zone absorbed ultra violet light and reduced blue tetrazolium. In addition, all of the zones found on the control chromatograms were seen. The chromatograms contained no suggestion of the conversion of 11-desoxycorticosterone to cortisol or aldosterone.

DISCUSSION

The rate of formation of formaldehydogenic substances from endogenous substrates by the adrenal cortices of rats of the Sherman strain, under the conditions of this study, was 14μg./100 mg. of adrenals/hr. This is approximately double the rates of 7, reported by us previously (3), and 6.7, found by Saffran and Bayliss (8), for the Sprague-Dawley strain of laboratory rat. Other than the difference in strains, no ready explanation for this discrepancy can be offered.

Approximately 26% of the formaldehydogenic material reacted with the Porter-Silber reagent. Since the paper chromatographic analyses did not clearly indicate the presence of any of the commonly occurring steroids having a dihydroxyacetone-type side chain, such as cortisol or cortisone, the compounds comprising the PSC cannot be identified. They may be resident in unidentified zones on the paper chromatograms. The possibility also exists that a portion of this reactive material is non-steroidal. In his study on *in vitro* steroidogenesis in the rat, Eisenstein (9) found that 43% of the material which absorbed maximally at 240 mμ reacted with the Porter-Silber reagent. It is difficult to compare his finding with that reported here for different analytical methods were used in each case to determine the total output of adrenocortical steroids.

Previous work (1, 3, 4, 13) has suggested that the adrenal cortex of the rat secretes principally corticosterone and, in addition, aldosterone and a varying number of unidentified compounds but no cortisol. The data reported here are in agreement with these prior findings. The largest quantity of reactive material on the paper chromatograms was contained in a zone having a mobility similar to that of corticosterone. The occurrence of a zone whose mobility is similar to that of aldosterone could be an indication of the secretion of this mineralocorticoid by the rat adrenal cortex, though the zone may represent other compounds as well. This zone may be the counterpart of Fraction 8 of Farrell and Lamus (10) and spot X of Hechter *et al.* (11) for in mobility and reactivity to various means of detection the three zones are quite similar. Though a zone was seen having a mobility similar to that of cortisol, the consistent failure of the material in this zone to reduce blue tetrazolium would indicate the absence of any significant quantities of cortisol. Most investigators have not been able to detect cortisol in rat adrenocortical secretions although it must be noted that Heard and his associates (12) have reported the occurrence of cortisol and cortisone, in addition to corticosterone, in their *in vitro* studies with rat adrenal glands.

The bulk of the available evidence, then, indicates that under the experimental conditions employed in the various studies, the adrenocortical secretions of the laboratory rat contain relatively small amounts of steroids hydroxylated at carbon 17. A similar situation appears to obtain in the case of the rabbit (1, 14), the albino mouse (15) and the steer (2). Hydroxylation of carbons 11 and 21 seems to proceed normally in these species but hydroxylation of carbon 17 seems to occur only at a very slow rate. A possible explanation for this state of affairs emerges from the data reported above on the *in vitro* hydroxylation of various steroid substrates by rat adrenal glands. The data in Table 2 indicate that progesterone and 11-desoxycorticosterone were more readily converted to PSC than were their 11-hydroxylated congeners. One could infer from these findings that prior hydroxylation of a 21-carbon steroid at carbon 11 impedes, in some fashion, subsequent hydroxylation of that compound at carbon 17 by rat adrenal glands. This suggests the hypothesis that the adrenal cortex of the rat contains a relative over-abundance of an "11β-hydroxylase" whose activity causes the early hydroxylation at carbon 11 of an immediate precursor of corticosterone. Such an hydroxylated precursor might be 11β-hydroxyprogesterone. This prior hydroxylation at carbon 11 would so hinder subsequent hydroxylation at carbon 17 that the ultimate product of steroidogenesis, secreted in the greatest quantities, would be corticosterone, while only small amounts of 17α-hydroxycorticosterone would be elaborated.

The experiments in which substrate concentrations were increased tenfold do not lend support to a possible hypthesis that hydroxylation at carbon 17 in the rat adrenal cortex is deficient because of an absolute

paucity of the requisite enzyme. The data in Table 2 suggest that the capacities of the hydroxylating enzymes were not exceeded when the substrate was supplied in a concentration of $10^{-4}M$ and that the rate of formation of PSC could increase with a large increase in substrate concentration. (This implies, of course, that the substrate supplied can be readily acted upon by the appropriate enzyme. A steroid already hydroxylated at carbon 11 may not be an example of such a substrate.) These findings tend to minimize the possibility of an enzyme deficiency being an important factor in the hydroxylation problem of the rat but certainly do not exclude it from consideration. As a first approximation, one could, of course, assume that an increase in the concentration of "17α-hydroxylase" in the rat adrenal cortex would increase the rate of hydroxylation at carbon 17. Kass *et al.* (14) in the rabbit and Rosenfeld (16) in the calf have been able to demonstrate an increase in the output of adrenocortical steroids having a dihydroxyacetone-type side chain following the administration of ACTH. These findings may reflect the correction of an enzyme deficiency or possibly the overcoming of an inhibition to hydroxylation; the explanation remains to be determined.

The data reported in this study tend to suggest that the hydroxylation problem in the rat adrenal cortex may stem more from a lack of appropriate substrate for the "17α-hydroxylase" to act upon than from a deficiency of the enzyme itself.

SUMMARY

In an effort to determine why the laboratory rat, unlike most other mammals studied, secretes primarily corticosterone rather than 17α-hydroxycorticosterone, a study of the ability of quartered rat adrenal glands to hydroxylate various 21-carbon steroids under *in vitro* conditions was undertaken. The conversion products were measured as formaldehydogenic substances and Porter-Silber chromogens and also subjected to analysis by paper chromatography. In comparing the results obtained when progesterone was added to those obtained with the use of 11β-hydroxyprogesterone and contrasting similarly the yields from the additions of 11-desoxycorticosterone and corticosterone, the data suggest that the prior hydroxylation of a 21-carbon steroid at carbon 11 hinders its subsequent hydroxylation at carbon 17 by the rat adrenal cortex. The hypothesis is thus suggested that a relative over-abundance of an 11-hydroxylating enzyme in the adrenal cortex of the rat impedes the elaboration, by this gland, of any large quantities of 17α-hydroxycorticosterone.

A ten-fold increase in substrate concentration revealed no enzymatic deficiency on the part of the rat to hydroxylate appropriate steroids at carbon 17, as determined by the anayltical methods used.

Acknowledgments

The author gratefully acknowledges the technical assistance of the Misses Flora Hernandez and Rhoda Nussbaum. The corticosterone used in this study was generously supplied by Dr. E. D. Nielson of the Upjohn Company and the aldosterone by Dr. R. Gaunt of Ciba Pharmaceutical Products, Inc.

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PRODUCTION OF EXOPHTHALMOS IN *FUNDULUS HETEROCLITUS* (VAR. BERMUDAE) BY TRIIODOTHYRONINE AND DESICCATED THYROID[1,2]

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THE usual clinical and experimental presumption has been that exophthalmos is due to an excess of a pituitary substance. Exophthalmos has been produced repeatedly in the thyroidectomized guinea pig by pituitary preparations (1 and 2). Desiccated thyroid (2) and thyroxine (1) are reported not to produce exophthalmos in this animal. Exophthalmos has also been produced by pituitary extract in *Fundulus heteroclitus* (3). The present studies were designed with the above background to test the clinical assumption that additional thyroid would antagonize the exophthalmos which occurred during treatment of thyrotoxicosis. However, the results were at variance with the above work.

EXPERIMENTAL METHODS

F. heteroclitus Bermudae were netted from Lover's Lake, St. George's West, Bermuda, during August 1956. This lake is slightly brackish. The fish at this time were probably near the end of their breeding season (personal communication H. Winn). The fish were then taken immediately to the laboratories of the Biological Station. Some of them were put in tanks of running sea water and others in aerated rain water. No differences were noted in the responses of these two groups, and they will not be separated in the discussion and presentation of results. The fish were kept in groups of 5 to 10, either in separate tanks or separated by wire netting from other groups and were fed daily on flaked anchovies. They were marked for individual recognition by a colored string through the lower lip, using the method of Dobyns (4). The material to be injected was dissolved in 0.1 to 0.2 cc. volume and injected via a No. 26 needle inserted through the cloaca into the peritoneal cavity. Exophthalmos was quantitated by determining the intercorneal distance daily for four days with a mechanic's caliper with a vernier scale by the method of Dobyns (4).

A control group was compared with groups given 0.2 mg. TSH[3] (Armour #317–52), 0.1, 0.2, 0.5 and 1.0 μg. triiodothyronine (T_3), and 0.6 mg. desiccated thyroid (Merrell) daily.

RESULTS

An increase in intercorneal diameter of .02 inch or more was chosen as the change to be considered as showing an effect. The results are listed in

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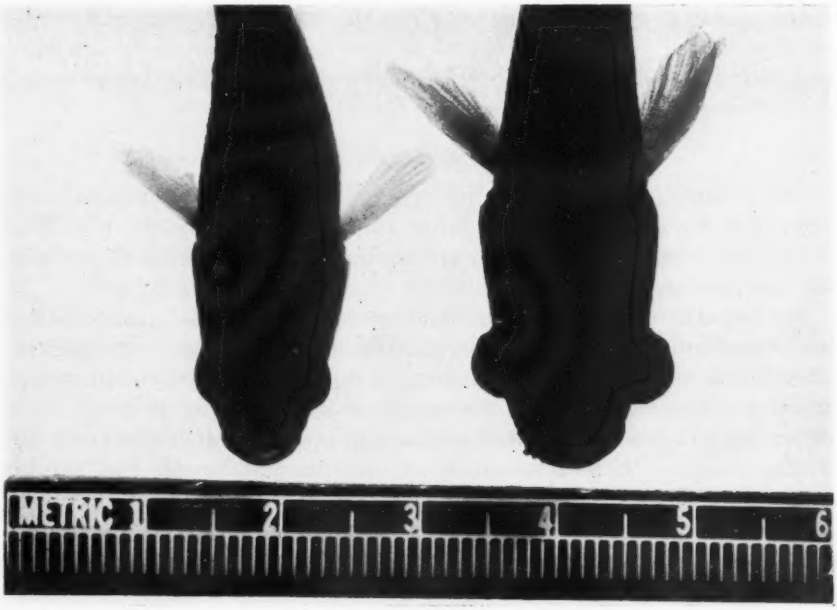

FIG. 1. Specimen injected daily for 7 days with 1.0 μg. T_3 on right. Control is on left.

Table 1. Figure 1 shows a fish injected daily with 1.0 μg. T_3 compared with a control. There was no significant difference between the control and TSH treated group. When the triiodothyronine and thyroid treated groups were lumped, there was a highly significant increase over either the control or TSH treated group ($p < 0.01$). There was no significant difference in the mean maximum response of any of the triiodothyronine treated groups with a range of .01–.07 inches increase.

An attempt was made to increase the dose of TSH, but the amount used, 0.4 mg. daily, killed all fish within 24 hours. The exophthalmos by visual inspection usually progressed equally, but there were four instances where one eye became definitely more prominent than the other during the

TABLE 1. EFFECT OF TSH, T_3, DESICCATED THYROID ON INTERCORNEAL DIAMETER COMPARED TO CONTROLS. THE NUMBER OF FISH IN EACH GROUP SHOWING MORE OR LESS THAN .02 INCHES INCREASE IN INTERCORNEAL DIAMETER IS LISTED

	Control	TSH, 0.2 mg. q.d.*	Doses of T_3 q.d.				Thyroid, 0.6 mg. q.d.
			0.1 μg.	0.2 μg.	0.5 μg.	1.0 μg.	
>.02″	1	1	2	5	4	4	4
<.02″	7	9	2	1	1	1	0

* q.d. = daily.

development of exophthalmos. Also, on the lower dose range the exophthalmos tended to develop after two to three days, last for one or two days, and then disappear despite continued therapy. In the higher dosage ranges it continued to persist and progress.

DISCUSSION

These results are in marked divergence to most of those reported in the literature. We do not place too much emphasis upon the failure of TSH to produce exophthalmos in this preparation, as it may be that we were not using an effective dose.

We feel that our results are unequivocal in that marked exophthalmos was produced in this species by both desiccated thyroid and triiodothyronine. This is certainly in contradistinction to the published statements in literature. However, despite the many studies of exophthalmos, it is surprising how few documented and carefully measured studies there are of the effect of thyroid preparations upon exophthalmos. The careful studies of Pochin (1) in guinea pigs include a statement that thyroxine 0.8 mg. daily produced no response. How long this therapy was continued was not stated. The studies of Marine (2) also refer to lack of effect of thyroid in producing exophthalmos in the guinea pig. No quantitative data were given. The failure of Albert (7) to produce exophthalmos in the Fundulus with thyroxine and thyroid substance is not at variance with the results reported here, as he used only one dose. LaRoche and LeBlond (5) did give thyroxine and desiccated thyroid to salmon and noticed apparent enopththalmos which was due to overgrowth of tissue around the eyes, but the amount of retroorbital tissue was increased, and apparently the distance between the lens was increased also. Repeatedly exophthalmos has been produced by pituitary extract in thyroidectomized guinea pigs (1, 2, and 6). Despite this, the results reported here suggest that the experimental production of exophthalmos be evaluated critically and that the exclusive role of the pituitary be subjected to especially searching scrutiny.

SUMMARY AND CONCLUSION

In the minnow, *Fundulus heteroclitus* Bermudae, exophthalmos was produced with triiodothyronine and desiccated thyroid, but not with a pituitary preparation.

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EFFECT OF STEROID DOSE AND STEROID RATIO ON PLATEAUING IN THE ADRENALECTOMIZED RAT RECEIVING A GROWTH HORMONE-CONTAINING PITUITARY EXTRACT

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CHRONIC treatment, with a constant dose per animal or per kg., of either purified bovine anterior pituitary growth hormone, or crude growth hormone containing extracts of bovine or rat pituitaries, fails to produce continuous growth, indefinitely, in either intact or hypophysectomized Norway rats (1, 2, 3, 4, 5). Treatment with growth hormone at a high dose level will give continuous growth for as long as a year and produce rats as large as 2.5 times normal adult size, yet plateauing still occurs (6, 7). That this phenomenon of plateauing on a constant dose of growth hormone is not merely a reflection of age changes in the animals is shown by the fact that senile rats, older than any used in any of the above mentioned experiments, show as great a growth response to growth hormone as do young adults (5). It has been shown by Selye that plateauing occurs at a higher weight level if DCA is given along with growth hormone in the intact rat (2), and that the initial growth response to growth hormone in the adrenalectomized rat requires either a mineralocorticoid, or NaCl in excess of that present in Purina Fox Chow (8).

We have previously postulated (4) that the altered growth response of rats plateaued while on growth hormone treatment, might be due to an altered ratio of anabolic to catabolic steroids. This, however, has been shown not to be the case by the data of Reinhardt and Li (9), who demonstrated flat plateaus in adrenalectomized, ovarectomized rats on NaCl replacement, receiving 0.6 mg. growth hormone/rat/day. When such animals were placed on 1.5 mg. growth hormone/rat/day, there was resumed growth, but at a rate of only about 1.0 gm./rat/day compared to the 3 to 4 gm. growth/rat/day in rats initially given a dose of this magnitude.

The present investigation was performed to determine: (1) If the adrenalectomized rat, on constant steroid replacement, would show the same type of plateauing as the intact rat; and (2) If growth plateaus in such animals would be sensitive to either the absolute level of steroid replacement or to the ratio of mineralocorticoid to glucocorticoid.

MATERIALS AND METHODS

Six-month-old female rats (Long-Evans Black) were adrenalectomized under ether anesthesia, using the lumbar approach. On the day of adrenalectomy, each rat was put

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on 200 μg. cortisone acetate[1]/rat/day. Any rat which lost 20% of its 24 hr. postoperative weight within 10 days, while on the 200 μg. cortisone treatment, was considered devoid of accessory adrenal tissue and satisfactory for the experiment. (Essentially 100% of rats meeting this criterion will die if mineralocorticoid or extra NaCl is indefinitely withheld). Following the experiment, all rats on which data are reported, again met this criterion.

After a rat had met the above criterion for completeness of adrenalectomy, it was put on 300 μg./day cortisone acetate plus 300 μg./day desoxycorticosterone acetate (DCA) administered as a single daily injection of a mixture of the microcrystalline suspensions of the steroids in 0.2% tween-80 (polyoxyethylene sorbitan mono-oleate) in isotonic saline solution. Injections were given subcutaneously along the dorsal surface, under aseptic conditions, and the rats were rewarded with 0.5 ml. imitation maple syrup following each injection.

The pituitary extract used was prepared by the method previously described for a pH 10.5 extract (4). All of the extract was prepared at the same time and stored at −30° C in injection vials until just prior to injection. The pituitary extract was injected subcutaneously, once daily, by the same technique used for the steroids. The daily dose of the extract given, during the first 140 days of treatment, contained the pH 10.5 soluble extractives of 25 mg. (wet weight) bovine anterior lobe tissue. This dosage is approximately equivalent to 0.8 mg./rat/day of growth hormone by weight gain in the 10-day normal rat assay.

The experiment was performed in a semi-isolation room in which the rats had been reared from birth. Room temperature was maintained at 22 to 24° C and Purina Laboratory Chow was fed *ad lib.*

Following addition of DCA to the replacement regimen, the weight lost was rapidly regained and plateauing occurred in an average of 12 days, at approximately the preoperative weight. The rats were divided into experimental and control groups by split litter technique and pituitary injections were begun in the experimental group on an average of 20 days following the addition of DCA to the replacement.

RESULTS

As in the intact rat, there was a period of rapid growth following the onset of treatment with the pituitary extract. This initial rapid growth was followed, again, just as in the intact rat, by attainment of a new growth plateau at a greater wight even though the dosage remained the same. The response during the first 40 days of treatment is shown in Figure 1.

A comparable group of intact rats receiving the same dosage of the same extract, run simultaneously, gained 48.5 ± 3.9 gm. over their controls during the first 16 days of treatment, compared to a gain of 45.5 ± 2.6 gm. over their controls by the adrenalectomized rats. From the 24th to the 40th day of treatment, the normal rats gained -4.3 ± 4.5 gm. over their controls, compared to 2.8 ± 3.4 gm. gained by the adrenalectomized treated rats over the adrenalectomized controls. In neither case is the difference between normal and adrenalectomized groups statistically meaningful.

From the 40th to 140th day of the experiment, the dosage of both the pituitary extract and the steroids was maintained at exactly the same level.

[1] Made available through the kindness of Dr. Elmer Alpert, Merck & Co., Inc. Rahway, N. J.

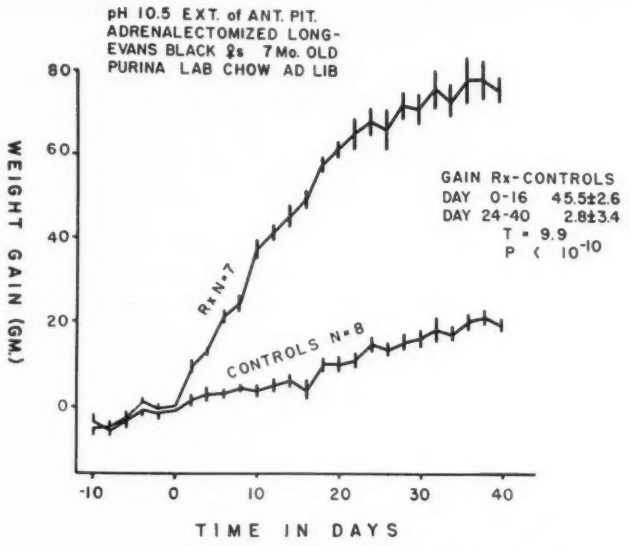

FIG. 1. Response of adrenalectomized rats to pH 10.5 extract of bovine anterior pituitaries. A constant daily dosage containing the pH 10.5 extractives of 25 mg. (wet weight) bovine anterior pituitaries/rat/day was given to the treated group from day 0 to day 40. This dosage is equivalent to 0.8 mg. growth hormone/rat/day. Steroid replacement was identical in the 2 groups.

The vertical bars extend 1 standard error of the mean above and 1 standard error below the mean value at each point. Gain of treated animals minus controls reported ±the standard error of the mean.

During this 100 day period, the growth hormone treated rats gained only 18.5 ± 9.5 gm. over their controls.

On the 140th day of the experiment, the dosage of the pituitary extract and both steroids was increased to make the dosage per unit body weight equal to that employed at the beginning of the experiment. As shown in Table 1, there was a slight resumption of growth with the increased dosage, followed again by a nearly flat growth plateau.

On the 196th day of the experiment, the dosage of both steroids was doubled. As shown in Table 1, this increase in steroid dosage had only a slight effect on growth of either the pituitary treated or control groups.

From the 212th day to the 228th day, the cortisone dosage was reduced to ⅓ the DCA dosage with again, only slight effect on the growth of either group as shown in Table 1.

DISCUSSION

We interpret the above data as indicating: 1) That adrenalectomized adult female rats, receiving 300 μg./rat/day cortisone acetate plus 300 μg./rat/day DCA plus a constant dosage of a growth hormone containing extract, plateau in essentially the same time and at essentially the same

TABLE 1. GROWTH DURING KEY 16-DAY PERIODS IN ADRENALECTOMIZED RATS TREATED WITH A pH 10.5 EXTRACT OF BOVINE ANTERIOR PITUITARIES

Experimental period days	Dosage			Gain, gm.		
	Pit. ext. mg.*/ kg./day	Cortisone, mg./ kg./day	DCA mg./ kg./day	Treated pit.steroids	Controls steroids only	Treated minus controls
0– 16	100.0	1.20	1.20	49.1 ± 2.2†	3.6 ± 1.4	45.5 ± 2.6
24– 40	83.0	1.00‡	1.00‡	8.3 ± 2.8	5.5 ± 1.9	2.8 ± 3.4
124–140	74.0	0.88‡	0.88†	4.0 ± 1.7	0.8 ± 1.3	3.2 ± 2.1
140–156	100.0	1.20	1.20	19.3 ± 2.6	1.5 ± 1.3	17.8 ± 2.9
180–196	100.0	1.20	1.20	6.3 ± 2.4	2.2 ± 1.3	4.1 ± 2.8
196–212	100.0	2.40	2.40	−5.6 ± 4.8	0.6 ± 1.9	−6.2 ± 5.2
212–228	100.0	0.80	2.40	5.7 ± 2.4	7.3 ± 1.3	−1.6 ± 2.7

* Dosage expressed as mg. (wet weight anterior pituitary tissue) represented by the daily dose.

† All means ± their standard errors.

‡ These steroid dosages apply to the pituitary group only. Since the controls gained an average of only 20.5 gm. during the first 140 days of the experiment, their dosage on a weight basis was essentially constant for the first 196 days.

weight as do intact adult females receiving the same growth hormone dosage. 2) The weight plateaus of rats at this dose level of the growth hormone containing extract are not appreciably altered by relatively large shifts in either the level of steroid dosage or in the ratio of glucocorticoid to mineralocorticoid. This would further indicate that a change in ratio of the endogenously produced glucocorticoid to mineralocorticoid is not an appreciable factor in plateauing on growth hormone *at least at this dose level.*

SUMMARY

Adrenalectomized adult female Long-Evans rats were maintained on 300 μg./rat/day cortisone acetate plus 300 μg./rat/day DCA. One group was given in addition a growth hormone containing extract equivalent to 0.8 mg. growth hormone/rat/day. The growth hormone treated animals developed a weight plateau in exactly the same manner, as intact growth hormone treated rats and maintained this plateau for 116 days, while on constant treatment. Doubling the dosage of both steroids, produced only a minor shift (loss) in the weight plateau. Likewise, changing the steroid ratio to 0.8 mg. cortisone/kg./day and 2.4 mg. DCA/kg./day gave only a very slight shift (gain) in the weight plateau.

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EFFECTS OF TESTOSTERONE ADMINISTRATION ON ACID SOLUBLE AND INSOLUBLE GLYCOGEN IN THE LEVATOR ANI MUSCLE[1]

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THERE is agreement in recent papers that testosterone is "glycopexic" and "glycostatic" with respect to several striated muscles (1–3). Early reports on the effects of testosterone on glycogen storage in striated muscles have been reviewed by Leonard (1).

Bloom, Lewis, Schumpert, and Shen (4) have shown that cold trichloroacetic acid (TCA) will consistently extract about 55% of the glycogen normally found in muscle following potassium hydroxide (KOH) digestion. These workers and others (5–9) have shown that tissue levels of TCA soluble and insoluble glycogen vary independently of each other under various physiological conditions. This has led to the concept that muscle glycogen may be comprised of two physiological entities.

The present study was undertaken to determine the effects of testosterone propionate (TP) on TCA-soluble and insoluble glycogen, and to determine the relationship between the glycogen changes and the phenomenon of TP induced growth of the levator ani muscle in the rat.

MATERIALS AND METHODS

Male rats of the Holtzman strain were maintained on Rockland Rat Diet and tap water, *ad libitum*, throughout the experiments. Testosterone propionate was administered subcutaneously in 0.1 ml. oil and control rats were not injected. In Experiment A, weanling rats weighing 40–45 gm. were castrated at 21 days-of-age and starting on the same day were injected daily with 0.1 mg. TP. In Experiment B, rats weighing 100–110 gm. were castrated at 40 days-of-age. Administration of 1 mg. TP per day was initiated 2 weeks after the operation. In both experiments groups of animals were sacrificed for muscle glycogen determinations following TP treatment for 1, 3, 5 and 7 days.

The procedure used to determine TCA-soluble glycogen was a modification of the method of Bloom, *et al.* (4). The animals were anesthetized with Nembutal and the muscles were removed rapidly and frozen between 2 small blocks of dry ice. The frozen muscles were weighed on a torsion balance and then homogenized in cold 10% TCA with a small glass homogenizer. The TCA-soluble glycogen was extracted in the cold for 2–4 hours with occasional shaking. The homogenate was then centrifuged at 4° C to separate the TCA-soluble glycogen of the supernatant from the TCA-insoluble glycogen in the precipitate. The TCA-soluble glycogen was precipitated with ethanol, hydrolyzed to glucose with 0.6N. hydrochloric acid, and determined as glucose by the

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method of Reinecke (10). The original precipitate containing the insoluble glycogen was hydrolyzed in hot 30% KOH. The TCA-insoluble glycogen was then precipitated with ethanol, hydrolyzed to glucose and analyzed as above.

The glycogen data are presented in terms of either glucose per muscle or glucose concentration per wet weight of muscle tissue. No significant changes were found in dry weight or total nitrogen concentration in the levator ani throughout these experiments, so the glycogen relationships remained unaffected by expressing the data in these ways.

Initially in Experiment A, TCA-soluble and -insoluble glycogen were determined using one-half of a muscle, and total glycogen determined in the other half. Subsequent analyses indicated that there was no difference between the total glycogen concentration values obtained directly and those obtained indirectly by adding the TCA-soluble and -insoluble values for a muscle. Thereafter TCA-soluble and -insoluble glycogen were determined on whole muscles and total glycogen concentration was obtained by adding the two values.

RESULTS

Tables 1 and 2 show absolute values for both control and treated groups. Figure 1 illustrates the comparison of the experimental results to the controls of the same day of treatment, since considerable random variation was observed in control glycogen concentrations on different days.

Experiment A, 21-day old rats

Figure 1-A shows that TP affected the TCA-soluble glycogen concentration more than the TCA-insoluble glycogen of the levator ani. There was

TABLE 1. EFFECT OF DAILY TP ADMINISTRATION ON GROWTH AND GLYCOGEN CONTENT OF THE LEVATOR ANI MUSCLE IN 21-DAY OLD RATS[1]

	TCA soluble glycogen, mg.% (glucose)			
	Day 1	Day 3	Day 5	Day 7
Control	249 ± 43[2]	365 ± 30	416 ± 57	351 ± 40
TP, 0.1 mg./day	399 ± 24	657 ± 63	405 ± 36	262 ± 31
	TCA insoluble glycogen, mg.% (glucose)			
	Day 1	Day 3	Day 5	Day 7
Control	333 ± 70	322 ± 36	393 ± 67	326 ± 27
TP, 0.1 mg./day	323 ± 20	308 ± 21	307 ± 11	242 ± 29
	Total glycogen, mg.% (glucose)			
	Day 1	Day 3	Day 5	Day 7
Control	551 ± 25	685 ± 38	843 ± 67	674 ± 37
TP, 0.1 mg./day	722 ± 35	951 ± 57	700 ± 23	535 ± 30
	Muscle weight, mg.			
	Day 1	Day 3	Day 5	Day 7
Control	6.4 ± 0.4	10.2 ± 0.9	12.6 ± 0.8	13.6 ± 0.4
TP, 0.1, mg./day	9.1 ± 0.7	15.7 ± 0.6	29.0 ± 1.3	39.1 ± 1.3

[1] Five to eight rats/group.
[2] Standard error.

TABLE 2. EFFECT OF DAILY TP ADMINISTRATION ON GROWTH AND GLYCOGEN CONTENT OF THE LEVATOR ANI MUSCLE IN 54 DAY OLD RATS[1]

	TCA soluble glycogen, mg.% (glucose)			
	Day 1	Day 3	Day 5	Day 7
Control	154 ± 18[2]	277 ± 34	262 ± 28	371 ± 44
TP, 1 mg./day	648 ± 38	698 ± 38	552 ± 23	423 ± 10
	TCA insoluble glycogen, mg.% (glucose)			
	Day 1	Day 3	Day 5	Day 7
Control	212 ± 17	228 ± 11	222 ± 27	247 ± 25
TP, 1 mg./day	280 ± 16	233 ± 28	238 ± 28	206 ± 19
	Total glycogen, mg.% (glucose)			
	Day 1	Day 3	Day 5	Day 7
Control	365	505	484	618
TP, 1 mg./day	928	930	790	629
	Muscle weight, mg.			
	Day 1	Day 3	Day 5	Day 7
Control	42.6 ± 2.5	48.2 ± 5.6	50.5 ± 3.9	44.4 ± 3.0
TP, 1 mg./day	54.9 ± 2.6	70.0 ± 3.6	91.4 ± 3.9	111.6 ± 2.4

[1] Six rats/group.
[2] Standard error.

a sharp rise in soluble glycogen during the early phase of TP administration which reached a peak following 3 days of treatment. As TP was continued, the concentration of soluble glycogen decreased to that found in controls on day 5 and slightly below the control level on day 7. Figure 1-A shows that the rapid depletion of TCA-soluble glycogen occurring between 3 and 7 days of TP treatment corresponded to the period of rapid growth of the muscle.

The effect of TP on levator ani TCA insoluble glycogen was not striking. There was no change in concentration after 1 to 3 days of treatment and a small decline after 5 to 7 days. It follows that the changes in total glycogen concentration (Table 1) will reflect mainly the changes in TCA-soluble glycogen.

Figure 1-A indicates that there was no change in TCA-soluble glycogen *per muscle* after 3 days of treatment despite the fact that the levator ani weight doubled between days 3 and 7. Insoluble glycogen per muscle was gradually increased from day 1 through day 7 of TP treatment, suggesting a parallel increase with muscle growth.

Experiment B, 54-day old rats

The second study, Experiment B, was designed to test the effects of TP on levator ani glycogen fractions in older animals which were given a 2 week rest period following castration. Table 2 contains the glycogen values

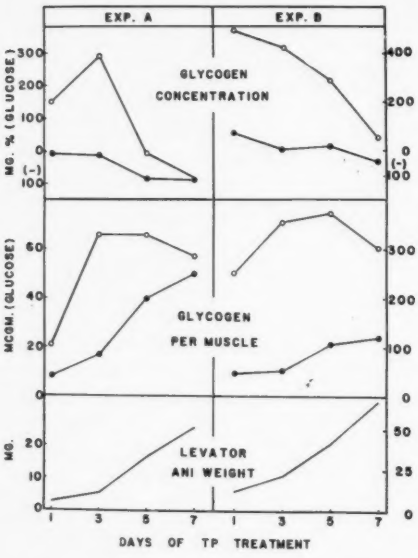

FIG. 1. Glycogen and weight changes in levator ani muscles following TP administration of 0.1 mg./day to 21-day-old rats (A) and 1.0 mg./day to 54-day-old rats (B). ○ and ● represent values for TCA-soluble and -insoluble glycogen respectively.

and muscle weights obtained in castrate 54-day old rats which were treated with 1 mg. TP per day for 1, 3, 5 and 7 days.

A larger absolute increase in TCA-soluble glycogen following TP administration was obtained in this experiment than in the 21-day old animals, but the trends in the two experiments were similar (*cf.* Fig. 1-A and B). In both Experiments A and B there were large increases in soluble glycogen following 1 and 3 days of TP administration. The increase subsided by day 5 in Experiment A and by day 7 in B, indicating that the TP-induced deposition of glycogen was maintained longer in the older than in the younger rats. Treatment with TP did not change the insoluble glycogen in the 54-day old rats (Fig. 1-B), which was similar to the results with the younger rats.

In Experiment B, TCA-soluble glycogen *per muscle* (Fig. 1-B) increased rapidly on the first day of treatment, but then leveled off after 3 days and showed no further increase from days 3 to 7 when the muscle was growing rapidly (Fig. 1-B). This was in complete agreement with the effect of TP on the 21-day old rats. Insoluble glycogen per muscle showed a gradual rise from day 1 to 7 in both Experiments A and B.

DISCUSSION

In general, TP induced similar glycogen changes in both the 21- and 54-day old rats. A sharp rise in soluble glycogen concentration was found after 1–3 days of TP treatment, but this increase was not sustained through days 5–7. Soluble glycogen per muscle reached a maximum at day 3 and there was no further increase with continued treatment in spite of dramatic muscle growth. Little or no changes in TCA-insoluble glycogen concentration were found. These data support the concept that the soluble fraction is, physiologically, the more labile fraction (4, 8, 9, 11, 12).

Experiments on P^{32} uptake by the levator ani, conducted concurrently on 32 animals from experiment A, were undertaken by Meyer and Harding (13). The uptake increased in animals treated with TP on days 1–3, but

it declined slightly below the controls on days 5–7. The results indicate that there was a striking parallel between the levator ani P^{32} uptake and the changes in TCA-soluble glycogen concentration in the TP-treated animals.

Since changes in levator ani glycogen, P^{32} uptake, and growth occur concurrently, consideration of a possible interrelationship between the processes is in order. A sharp rise in glycogen levels and P^{32} uptake was found when the muscle was starting to grow. However, following continued treatment rapid muscular growth was associated with a decreased uptake of P^{32} and a reduction in glycogen concentration. It is tempting to speculate that during TP treatment the early rises in soluble glycogen and P^{32} uptake represent a storage of potential energy (glycogen and high energy phosphate) which is subsequently depleted in the course of rapid protein synthesis and accelerated muscle growth.

Levator ani growth is gradual and glycogen is low in both castrate adult and normal immature rats. Possibly the low glycogen level is adequate for normal metabolism and growth, whereas the TP induced increase in glycogen makes rapid growth possible. A recent paper by Leonard (2) shows that certain muscles such as the cremaster are more sensitive morphologically to TP than the leg and abdominal muscles. In addition, castration for 7 days causes a decrease in cremaster glycogen, but this reduction in the less senstive muscles of the leg and abdomen was apparent only after 32 days. These data suggest that muscle glycogen may be related to growth. The protein sparing action of carbohydrate (14) may also implicate glycogen as a contributor of energy for muscle growth.

Leonard (2) has suggested that glycogen storage induced by TP may be another manifestation of the anabolic property of the steroid. Possibly the protein anabolic action of TP is mediated via a primary effect on the energy (carbohydrate) aspects of muscle metabolism. The mechanisms involved in protein synthesis may be accelerated when extra energy is made available in the form of increased TCA-soluble glycogen levels.

SUMMARY

The effects of TP administration on the glycogen content of the levator ani muscle have been studied for 1, 3, 5 and 7 days in 21-and 54-day old rats. Initially, striking increases in glycogen concentration occur. However, with continued TP treatment, a decrease in glycogen concentration in the muscle was associated with rapid growth of the levator ani. These changes in total glycogen were found to be due entirely to the trichloroacetic acid soluble moiety, while acid insoluble glycogen remained unchanged. A possible mechanism for protein anabolic action of TP is discussed.

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HORMONAL FACTORS INFLUENCING CALORIGENESIS[1]

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IT IS well established that the thyroid is the gland controlling respiratory metabolism; that the thyroid in turn is controlled by a specific hormone of the anterior pituitary is equally well accepted. However, several reports have appeared which suggest that increase in metabolic rate can be obtained by pituitary extracts without thyroid mediation. Lactogenic hormone was reported to increase heat production in thyroidectomized as well as hypophysectomized birds (1). Growth hormone preparations were reported to increase the metabolic rate of thyroidectomized mammals (2, 3, 4). Evidence has been presented by Collip and his associates for a specific metabolic factor in anterior pituitary extracts other than thyrotrophic hormone (5, 6, 7). A calorigenic action from adrenocorticotrophic fractions has been reported by Astwood and others (8–14). Erythropoietically active pituitary fractions have been reported to restore the low metabolic rate of hypophysectomized and of thyroidectomized rats (15). A reexamination of the role of the pituitary and its target organs in calorigenesis was, therefore, in order.

MATERIALS AND METHODS

In this investigation the standard methods of deprival and replacement have been used. Hypophysectomy, thyroidectomy, adrenalectomy, gonadectomy, and combinations thereof were performed, and the thyrotrophic, adrenocorticotrophic, melanocyte-stimulating and lactogenic hormones of the pituitary were examined. The target organ hormones whose effects on calorigenesis were examined were cortisone, hydrocortisone, thyroxine, and triiodothyronine. Female rats of the Long-Evans strain, 26 to 28 days-of-age, were employed. Hormonal treatment was begun 45 days after operative intervention and was continued 12 days unless otherwise indicated. Operative procedures, criteria of completeness of ablations, care of animals, and types of hormones injected are given in more detail in the appendix (16–24).

The metabolic rates were determined eight hours after the last hormone injection with a closed circuit respiration apparatus consisting of eight chambers which was patterned after that described by Kleiber (25). All rats were fasted four hours, then allowed to adjust to the temperature of the apparatus ($30 \pm 0.1°$ C) for one hour prior to oxygen consumption measurements. The liters of oxygen consumed per rat over a three-hour experimental period were converted into Calories per hour, by the factor 4.80.

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The Calories per hour per square meter of body surface were calculated by Benedict's formula for the rat:

$$\text{Area in } m^2 = \frac{9.0 \times \sqrt[3]{(\text{body weight in gm.})^2}}{10{,}000}$$

The metabolic rate so described is the standard metabolic rate and not the basal metabolic rate as defined for human beings.

RESULTS

Deprival

The metabolic rates of rats deprived of pituitary, thyroid, adrenals or ovaries were compared with those of normal rats (Fig. 1). In evaluating the change in metabolic rate with increasing postoperative period the gradual decline in the metabolic rate of the normal rat with age (which eventually stablized at the fifth month of life at 30.3 Cal./m.²/hr.) had to be taken into account. No change in calorigenesis followed ovariectomy. Adrenalectomized rats showed a 10% decrease in metabolic rate soon after operation which was not progressive during the observation period. This was only true of rats given some form of maintenance therapy, otherwise there was a rapid fall in metabolism culminating in death. Following hypophy-

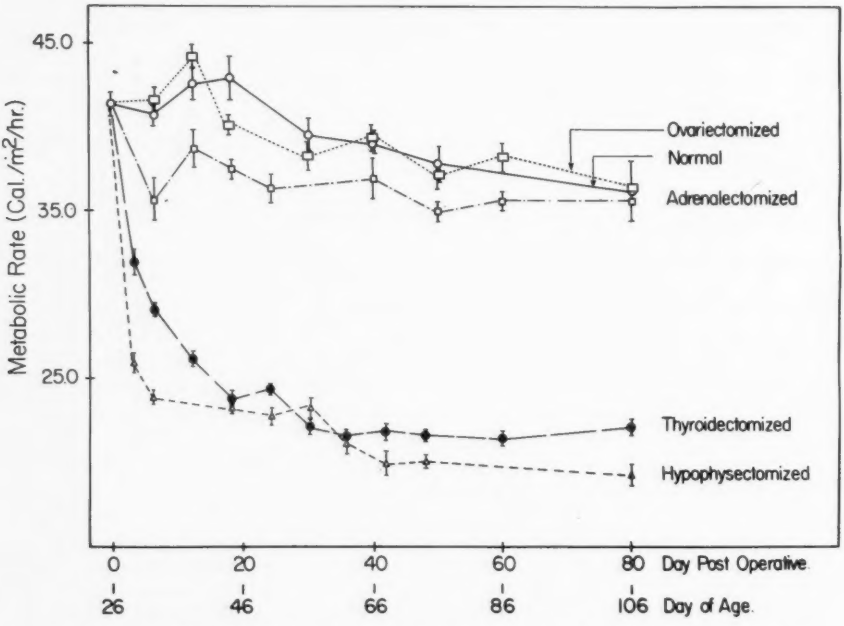

FIG. 1. The effect of ablation of endocrine organs on the metabolic rate of the rat. Each point on these curves was based on determinations employing 20 to 30 rats. The vertical bars extending from the points on the curves in all figures represent the standard errors of the means.

sectomy the decline in metabolic rate was more rapid than after thyroidectomy. Twenty-four hours after operation the metabolic rate of the hypophysectomized rats decreased 10 Calories while the thyroidectomized rats showed a decrease of only 3 Calories from the control level. After two weeks calorigenesis in the thyroidectomized rats approached the hypophysectomized level.

Replacement

Thyrotrophic and thyroid hormone:

The metabolic rate of hypophysectomized rats was not restored to normal by the highest dose of thyrotrophic hormone administered (Table 1).

TABLE 1. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED AND OF THYROIDECTOMIZED RATS TO THYROTROPHIN

Group	Dose TSH, μg./day/12 days	No. of rats	Metabolic rate, Cal./m.²/hr.	24 hr. I^{131} uptake†
$\overline{H}$	5	6	22.1 ±0.8*	—
	10	5	25.1 ±0.9	—
	50	6	27.0 ±1.3	—
Thyrotrophin	150	6	27.4 ±0.9	4.5 ±0.5
	300	6	29.0 ±0.8	6.0 ±0.8
	450	6	30.5 ±1.2	6.9 ±0.5
	500	8	33.9 ±1.2	8.0 ±2.0
$\overline{T}$	500	8	22.3 ±0.3	0.01 ±0.1
$\overline{H}$ Controls	—	25	22.6 ±0.6	0.5 ±0.1
$\overline{T}$ Controls	—	8	21.8 ±0.4	0.01 ±0.1
N Controls	—	19	41.8 ±1.5	8.5 ±1.4

* Figures preceded by ± in tables and text are the standard errors of the means.
† Expressed as per cent of injected dose in all tables.

I^{131} uptake by the thyroid was normal at this high level. Histologically the thyroids were hyperactive (Fig. 2), although the weight of the glands was not increased.[2]

In order to test the possibility that thyrotrophic hormone increases the metabolic rate independently of the thyroid gland, thyroidectomized rats were injected with thyrotrophic hormone. The thyroidectomized rats gave no calorigenic response and no significant I^{131} accumulation as shown in Table 1.

Assuming the thyroid gland to be the principal regulator of the metabolic rate, and the calorigenic decrease occasioned by hypophysectomy to be

[2] The metabolic rate, I^{131} uptake and histology of the thyroids returned to hypophysectomized control levels when injections were continued for 45 days. It is of interest that injection of the thyrotrophic preparation did not produce exophthalmos in hypophysectomized or normal rats, or in normal immature guinea pigs, when administered chronically in doses 200 times the minimal effective dose in the hypophysectomized rat.

only a consequence of the accompanying thyroid atrophy, then the quantity of thyroid hormone required to restore the metabolic rate of the hypophysectomized rat to normal should be the same as that required in the thyroidectomized rat. To test this assumption groups of rats were treated as follows:

Fourteen hypophysectomized rats were divided into two equal groups. One group received thyroxine in increasing doses from 1 μg. to 10 μg. over a 37 day period. The second group received 1 μg. of triiodothyronine

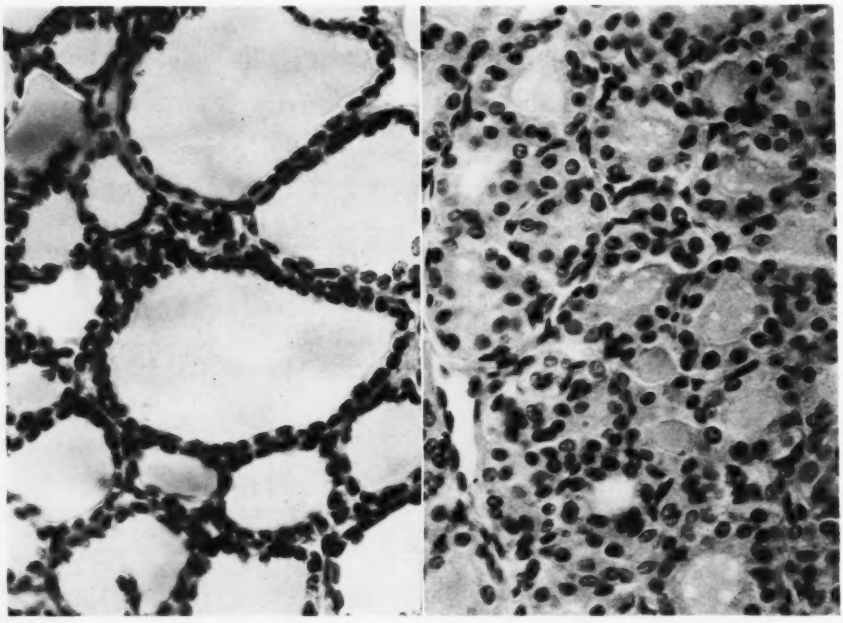

FIG. 2. Thyroid of hypophysectomized rats. H. and E. ×745. Left: Untreated hypophysectomized rat. Right: From hypophysectomized rat injected with 450 μg. of TSH daily for 12 days.

daily for the first 13 days, thereafter the dose was increased to 2 μg. for the remainder of the 37-day injection period. Sixteen thyroidectomized rats were divided into two equal groups, one receiving thyroxine in gradually increasing doses from 1 to 5 μg. and the other group 1 μg. of triiodothyronine for the entire 37-day injection period. Twenty intact rats served as untreated controls. Both thyroxine and triiodothyronine were more effective in thyroidectomized rats than in hypophysectomized rats (Fig. 3).[3]

[3] If the administered quantity of thyroxine or triiodothyronine were expressed on the basis of body weight, they were much more effective in thyroidectomized than in hypophysectomized rats inasmuch as the thyroidectomized rats were larger at onset of the injection period and the injections reinstated a normal growth rate. Growth rate

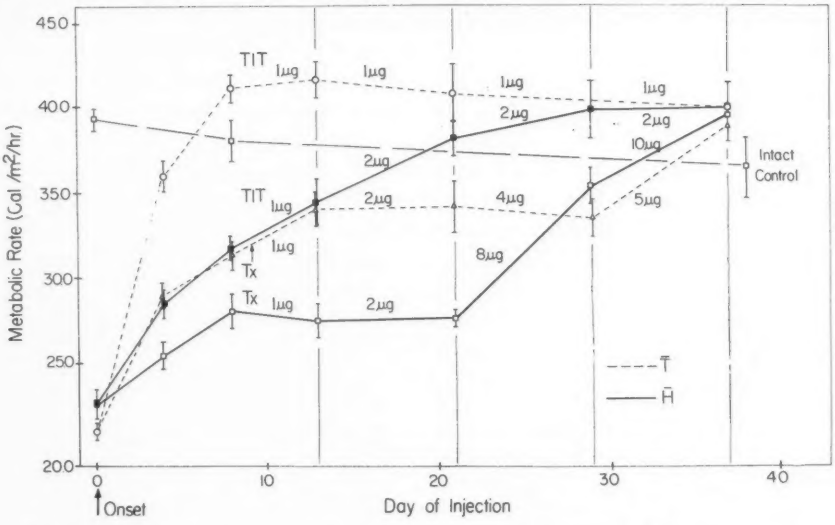

FIG. 3. Calorigenic response of thyroidectomized and of hypophysectomized rats to thyroxine and to triiodothyronine.

TABLE 2. INCREASED CALORIGENESIS IN HYPOPHYSECTOMIZED RATS INJECTED WITH ADRENOCORTICOTROPHIC PREPARATIONS

Preparation injected	Dose, µg./day/12 days	No. of rats	Metabolic rate Cal./m.²/hr.	Adrenals, mg.	24 hr. I^{131} uptake
Li α-corticotropin	50	6	36.9±1.7	35	1.0±0.2
Lederle α-pool corticotropin	100	12	31.1±1.0	26	—
Merck corticotropin B	50	7	36.9±1.6	17	—
	100	7	36.1±1.4	24	—
Li fraction E	200	6	35.5±1.1	30	1.8±0.3
Oxycellulose eluate	150	6	35.8±1.3	32	1.8±0.2
H̄ Controls	—	6	19.3±0.8	7	0.4±0.2
N Controls	—	19	41.8±1.5	73	8.5±1.4

Adrenocorticotrophic hormone:

Adrenocorticotrophic hormone significantly increased the metabolic rate of rats hypophysectomized, thyroidectomized or both hypophysectomized and thyroidectomized. The marked calorigenic response of hypophysectomized rats to adrenocorticotrophic preparations is presented in Table 2. The dose-response relationship of hypophysectomized rats to the highly purified α-corticotropin is presented in Table 3. All ACTH pre-

in thyroidectomized rats was brought back to normal at doses lower than those required to reinstate normal metabolic rate. It is well known that only low doses of thyroixne are needed to reinstate secretion from the pituitary of the thyroidectomized rat. Thyroxine caused negligible body weight increment in the hypophysectomized rats.

parations not only stimulated heat production but also restored body temperature, muscle tone, food intake and spontaneous activity to normal. The highest dose level of α-corticotropin produced a six-fold increase in water intake and urine excretion. The urine was of low specific gravity and contained no sugar. Body weight loss did not occur during the administration of the preparations. It should be noted that on a dosage basis expressed in mg. these adrenotrophic preparations were more efficacious than the thyrotrophic preparations in raising the low metabolic rate of hypophysectomized rats. Some thyroid stimulation resulted from administration of adrenotrophic preparations, for example, α-corticotropin caused detectable morphological evidence of stimulation and significantly increased I^{131} uptake at the 25 and 50 μg. doses (Table 3).

In order to eliminate the possibility that increased food consumption

TABLE 3. CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED RATS TO GRADED DOSES OF α-CORTICOTROPIN

Group	Dose, μg./day	No. of rats	Metabolic rate—Cal./m.²/hr.				Adrenals, mg.	24 hr. I^{131} uptake
			Day of injection					
			0	4	8	12		
α-corticotropin	5	6	18.3±0.6	20.5±0.4	22.2±0.7	20.1±0.6	9	0.5±0.1
	10	6	19.8±0.6	24.0±1.8	27.6±0.8	24.4±1.9	12	1.3±0.3
	25	6	20.6±0.8	26.5±1.3	32.5±1.7	31.1±1.5	25	2.2±0.4
	50	6	19.8±0.6	30.2±2.6	36.7±1.5	36.9±1.7	35	1.0±0.2
H Controls	—	6	18.0±0.7	20.3±1.0	20.9±1.0	19.0±0.9	6	0.4±0.2
N Controls	—	19	—	—	—	41.8±1.5	73	8.5±1.4

resulting from the injection of ACTH mediated the increased metabolic rate, the calorigenic action of ACTH was measured in hypophysectomized rats in which food intake was restricted to pretreatment levels. Twelve rats, 27 days postoperative, were divided into two equal groups. The average daily food consumption was measured for 11 days and the metabolic rate was determined twice during this control period. Injections of an adrenocorticotrophic preparation (oxycellulose eluate) were then given in 150 μg. daily doses for 13 days. One group was continued on the pre-injection average daily food consumption, whereas the second group continued to eat as desired. The results are presented in Figure 4. The food intake of the injected group allowed to eat at will increased from 5.4±0.1 gm. to 9.1±0.4 gm. During the same interval the metabolic rate increased from 23.3±1.2 Cal./m.²/hr. to 35.8 Cal./m.²/hr. The second group, although restricted to pre-injection food intake, showed the same increase in oxygen consumption. The severity of food restriction was evidenced by the immediate increase in food consumption, 2.5 gm., when the rats were allowed free access to food. The increased heat production occasioned by ACTH in rats on the restricted diet occurred at the expense of body weight. After the injections were terminated the metabolic rate and food intake of each group returned to pretreatment levels within seven days.

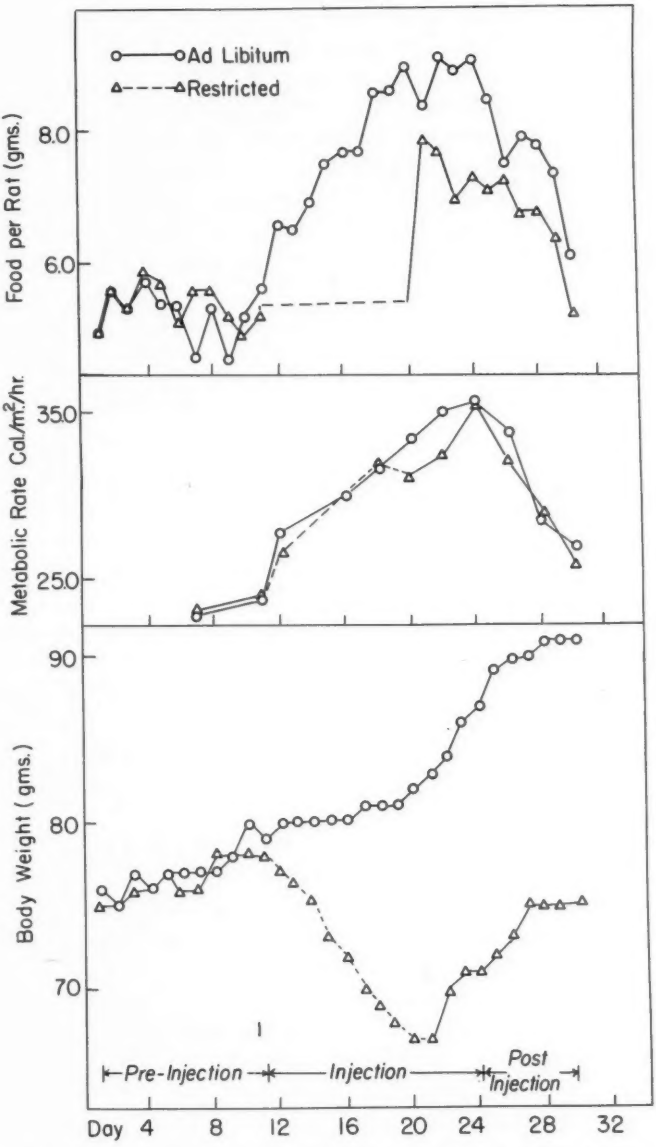

FIG. 4. Food intake, metabolic rate and body weights of hypophysectomized rats injected daily with 150μg. of oxycellulose eluate ACTH under conditions of food restriction.

If an increase in calorigenesis in hypophysectomized rats occasioned by ACTH also characterized the thyroidectomized rat, the metabolic increase could not be interpreted as mediated through the thyroid gland. In order to test this point, thyroidectomized rats (95 days postoperative) were injected with the oxycellulose eluate. The low metabolic rate characteristic of thyroidectomy was definitely increased but not restored to normal levels by the ACTH preparation (Table 4). A calorigenic action of ACTH (fraction E) was also observed in hypophysectomized-thyroidectomized rats but again was of less magnitude than in hypophysectomized rats (Table 4). An average eight gram body weight loss occurred both in

TABLE 4. INCREASED CALORIGENESIS IN THYROIDECTOMIZED AND HYPOPHYSECTOMIZED-THYROIDECTOMIZED RATS INJECTED WITH ACTH

Group	Dose, μg./day/12 days	No. of rats	Metabolic rate, Cal./m.²/hr.	Adrenals, mg.
Oxycellulose Eluate				
$\overline{T}$*	200	10	28.2±1.0	72
$\overline{T}$ Control	—	5	18.6±0.6	16
N Control	—	16	36.5±1.8	73
Fraction E				
$\overline{H}$–T†	250	4	34.1±1.8	86
$\overline{H}$†	250	6	37.8±1.8	106
$\overline{H}$ Control	—	31	25.6±0.6	9
N Control	—	18	42.8±0.8	20

* Ninety-five days post-operative at onset.
† Three days post-hypophysectomy and one day post-thyroidectomy at onset.

thyroidectomized and in hypophysectomized-thyroidectomized rats, whereas at the doses used the hypophysectomized rats maintained a stable body weight during the injection periods.

If ACTH were still effective in the repair of the low metabolic rate of the thyroidectomized rat in the absence of the adrenal, its action could be interpreted as due to an extra-adrenal effect of ACTH or it could still be interpreted as due to a specific metabolic hormone contaminating the ACTH preparations. Rats in which a low metabolic rate had resulted from thyroidectomy were also adrenalectomized and the restorative action of ACTH tested. The doubly operated animals were maintained on either NaCl, or hydrocortisone and desoxycorticosterone. One percent NaCl, although adequate to maintain adrenalectomized rats, proved unsatisfactory for thyroidectomized-adrenalectomized rats as evidenced by the failure of any animal to survive for over 50 days after the operation. The doubly operated animals maintained on salt (18 days postoperative) showed no increase in calorigenesis on injection of the oxycellulose eluate (Table 5). The increase in calorigenesis from this preparation in hypophysectomized rats was definite. Table 5 shows that ACTH (fraction E)

TABLE 5. ABSENCE OF CALORIGENIC RESPONSE OF THYROIDECTOMIZED-ADRENALECTOMIZED RATS TO ACTH

Group	Dose, μg./day/12 days	No. of rats	Metabolic rate, Cal./m.²/hr.	
			Initial	Final
Oxycellulose Eluate				
$\overline{T}$–$\overline{A}$*	200	4	22.5±0.3	23.6±0.7
$\overline{H}$	150	8	20.1±0.8	35.5±1.4
$\overline{T}$–$\overline{A}$ Control	—	3	21.0±2.9	23.8±2.4
$\overline{H}$ Control	—	8	23.2±0.6	21.2±0.6
Fraction E				
$\overline{T}$–$\overline{A}$†	250	6	25.7±1.0	27.9±0.8
$\overline{H}$	250	7	23.9±0.9	40.9±1.9
$\overline{T}$–$\overline{A}$ Control	—	6	25.4±0.5	25.0±0.7
$\overline{H}$ Control	—	6	20.5±1.0	21.0±0.9

* Maintained by 1% NaCl.
† Maintained by 0.1 mg. hydrocortisone acetate and 0.1 mg. desoxycorticosterone acetate.

given thyroidectomized-adrenalectomized rats maintained on adrenal steroids for 30 days was similarly ineffective in increasing calorigenesis although active in hypophysectomized rats.

Melanocyte-stimulating and lactogenic hormones:

O'Donovan and Collip (6) reported that the chemical and biological characteristics of the specific metabolic factor closely resembled those of the melanocyte-stimulating hormone. Since all highly purified ACTH preparations possess some melanocyte-stimulating properties, it has not been possible to differentiate between the calorigenic action of the two substances until the recent purification of a melanocyte-stimulating hormone free of adrenocorticotrophic properties (22). The data presented demonstrate that this hormone did not increase calorigenesis in hypophysectomized rats at the dose level employed (Table 6).

Lactogenic hormone did not cause significant change in the low metabolic rate of hypophysectomized rats at the dose levels employed (Table 6).

Growth hormone has been observed to enhance the thyroid stimulating action of thyrotrophin and to increase the calorigenic action of thyroxine

TABLE 6. ABSENCE OF CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED RATS TO MELANOCYTE-STIMULATING AND LACTOGENIC HORMONES

Hormone injected	Dose, μg./day/12 days	No. of rats	Metabolic rate, Cal./m.²/hr.
Melanocyte-Stimulating	100	5	19.8±0.9
Lactogenic	50	4	23.5±1.2
	1000	4	20.2±1.1
$\overline{H}$ Control	—	6	20.0±1.0
N Control	—	19	41.8±1.5

in the hypophysectomized rat. This data will be presented in a separate communication.

Hydrocortisone and cortisone:

Webster, Pfiffner and Swingle observed that adrenal cortical extract raised the metabolic rate of thyroidectomized cats between 15 and 30% (26). Cortisone has been reported to increase basal oxygen consumption in myxedema (12, 27). In order to reexamine the influence of the adrenal steroids on the gaseous metabolism in the absence of the thyroid, two groups of thyroidectomized rats when 120 days postoperative were in-

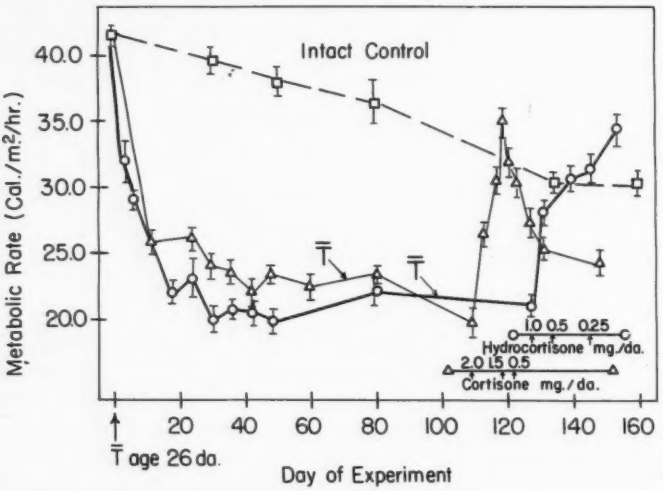

FIG. 5. Calorigenic response of thyroidectomized rats to hydrocortisone and to cortisone.

jected with either hydrocortisone or cortisone. Hydrocortisone was administered to a group of five rats and its influence on oxygen consumption was measured. As this substance at the initially high daily dose of 1.0 mg. caused loss of body weight, the dose was gradually decreased during the 27 day injection period to 0.25 mg. Cortisone was tested in a group of seven thyroidectomized rats and again its was necessary to decrease the dose from 2.0 mg. to 0.5 mg. during the 40 day injection period. Twenty intact rats served as uninjected controls. The results are presented in Figure 5. On injection of hydrocortisone the metabolic rate returned immediately to normal and the lowest dose level administered was still capable of maintaining it at normal levels. Cortisone at the higher dose level also restored the metabolic rate to normal but the calorigenic response decreased when the dose was reduced.

Combination of thyrotrophic hormone or thyroxine and hydrocortisone:

As the thyrotrophic hormone did not restore the metabolic rate of hypophysectomized rats, even though injected at high doses, and as adreno-

corticotrophic hormone and adrenal steroids were calorigenic, it was considered possible that the deficient adrenal cortical activity in hypophysectomized rats might explain the incomplete restoration of calorigenesis by thyrotrophic hormone. Twelve hypophysectomized rats when 24 days postoperative were distributed into equal groups and treated daily as follows: 1.0 mg. hydrocortisone (compound F); 450 μg. thyrotrophic hormone (TSH); 1.0 mg. hydrocortisone plus 450 μg. thyrotrophic hormone

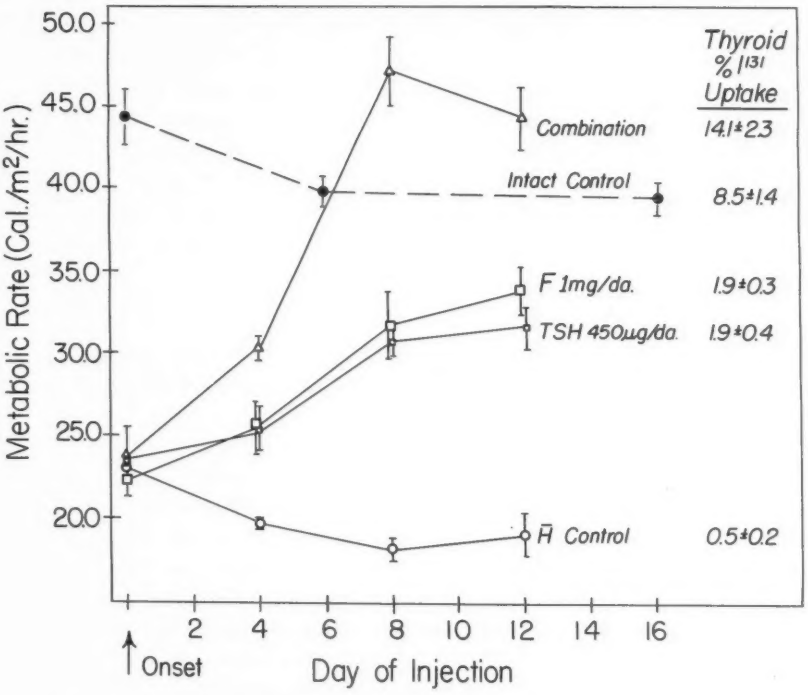

FIG. 6. Calorigenic response of hypophysectomized rats to hydrocortisone and thyrotrophic hormone alone and in combination.

(F+TSH). Four hypophysectomized and twenty intact rats were maintained as untreated controls. Injections were continued for 14 days. TSH and hydrocortisone exerted an additive effect on the metabolic rate of hypophysectomized rats (Fig. 6). Hydrocortisone also enhanced the activity of the thyrotrophic hormone on I^{131} uptake by the thyroid. Histologically the thyroids also showed greater activity in the hypophysectomized rats receiving the combined therapy, the thyroids of rats receiving TSH alone being within normal limits whereas the combination resulted in hyperplastic glands. The weights of the thyroids were not increased by the separate or combined treatment.

Thyroxine, as demonstrated, was less effective calorigenically in hypo-

TABLE 7. ADDITIVE CALORIGENIC RESPONSE OF HYPOPHYSECTOMIZED RATS TO HYDROCORTISONE AND THYROXINE

Treatment	Dose, μg./day	No. of rats	Metabolic rate—Cal./m.²/hr.		
			Day of injection		
			0	5	10
Hydrocortisone	500	6	21.8±0.8	26.0±0.8	29.9±1.1
Thyroxine	5	6	22.0±1.1	34.0±1.1	35.2±0.7
Combination	500+5	6	22.2±0.4	37.3±1.0	43.4±0.8
H Controls	—	6	22.1±0.9	20.5±0.5	21.2±0.4
N Controls	—	19	—	—	39.6±0.9

physectomized than in thyroidectomized rats. The restoration of normal adrenal function by thyroxine in the thyroidectomized rat, mediated through the anterior pituitary, possibly explains this difference. In experiments where hydrocortisone was administered to hypophysectomized rats this deficiency was repaired and the calorigenic response to TSH increased. Similarly, the effectiveness of thyroxine could be increased by hydrocortisone (Table 7).

DISCUSSION

The purpose of this study was to determine whether the increased heat production, which occurs in the absence of the thyroid following the administration of pituitary fractions, is mediated through one of the well known trophic hormones or through a specific metabolic factor. It was found that adrenocorticotrophic hormone was the only pituitary hormone tested which increased oxygen consumption in the absence of the thyroid. The ineffectiveness of adrenocorticotrophic preparations in the absence of the adrenals made untenable either the assumption that increased calorigenesis was due to an extra-adrenal action, or that adrenocorticotrophic hormone was contaminated by a specific metabolic factor.

Although it was clear that the adrenal steroids markedly increased calorigenesis in the absence of the thyroid, their role in heat production in the intact animal was not adequately understood. It is difficult to clarify this problem by the classic methods of deprival and replacement inasmuch as the adrenal gland is essential for life, and the decrease in calorigenesis which occurs following adrenal ablation cannot be dissociated from pathological changes in the ensuing moribund state. On the other hand adrenalectomized animals maintained on salt show only slight impairment of metabolism.

It is possible, however, to create a partial deficiency of adrenal cortical secretion which is compatible with continued life, in that the hypophysectomized rat has a subnormal but not completely atrophic adrenal. The metabolic rate declined rapidly after hypophysectomy, more rapidly even than after complete thyroidectomy. Thyrotrophic hormone administered to the hypophysectomized rat, in doses sufficient to produce histological

evidence of thyroid hyperactivity and normal I^{131} uptake, increased but did not restore the metabolic rate of the hypophysectomized rat to normal. The thyroid hormone was calorigenically more effective in the thyroidectomized rat. Another factor must therefore be active in the thyroxine treated thyroidectomized rat than in the hypophysectomized rat so treated. Neither the thyrotrophic hormone, nor the thyroid hormone influenced the atrophic adrenals of the hypophysectomized rats, whereas the thyroid hormone in thyroidectomized rats restored the adrenals. The role of adrenal steroids, in animals treated with thyrotrophic hormone or thyroxine, in determining the extent of calorigenesis must therefore be supplementary.

The observation that both adrenocorticotrophic preparations and hydrocortisone produced some thyroid stimulation in the absence of the pituitary, and that hydrocortisone remarkably enhanced the action of concurrently administered thyrotrophic hormone, suggest that a part of the calorigenic action of the adrenal cortex is mediated through the thyroid. These findings require confirmation for they are in direct contradiction to many studies which have shown that, in the hypophysectomized rat, cortisone alone has no effect on I^{131} accumulation by the thyroid and that it depresses the action of thyrotrophic hormone administered concurrently (28, 29, 30, 31, 32).

SUMMARY

Experimental evidence has been presented which supports the concept that the extra-thyroidal calorigenic action of pituitary fractions is attributable to adrenocorticotrophic hormone. The action is indirect, through adrenal steroid production, and is of fundamental importance in the control of respiratory metabolism.

The decline in metabolic rate after hypophysectomy was more abrupt than after thyroidectomy, and the calorigenic response of the hypophysectomized rat to thyroxine was less sensitive than that of the thyroidectomized rat. Both observations suggest that some calorigenic factor, in addition to the thyroid hormone, is acting in the thyroidectomized rats. High doses of thyrotrophic hormone, inadequate to restore the metabolic rate of hypophysectomized rats to normal, did so on simultaneous administration of hydrocortisone. All these findings indicate that adrenal steroids as well as the thyroid hormone contribute to normal heat production.

Adrenocorticotrophic hormone increased calorigenesis in the absence of the thyroid, but was only effective in the presence of the adrenals.

The restoration of thyroid function in hypophysectomized rats by thyrotrophic hormone was enhanced by hydrocortisone.

It would appear, therefore, that the thyroid hormone and adrenal steroids act conjointly in control of metabolic rate, but can act as independent

factors in determining the level of calorigenesis. Adrenal steroids may in addition influence metabolic rate through augmentation of thyroid acivity. The relative importance of these various factors in calorigenic homeostasis is still to be determined.

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APPENDIX

All rats were fed a standard laboratory diet consisting of 67.5% wheat, 15% casein, 7.5% skim milk powder, 6.75% hydrogenated vegetable oil. 1% fish oil, 0.75% NaCl, 1.5 $CaCO_3$, KI added (analysis 0.9–1.0 μg. iodine per gram of diet). They were kept at a constant temperature of $75 \pm 2°$ F. Following the surgical procedures the animals were injected intraperitoneally, for two consecutive days, with 1 cc. of 0.5% Terramycin in 5% glucose solution per 100 gm. body weight.

Hypophysectomy was performed through the parapharyngeal approach. In addition to the standard laboratory diet, fed dry, hypophysectomized and hypophysectomized-thyroidectomized rats received a wet mash of the same diet every afternoon. Rats were selected for experimentation on the basis of an immediate cessation of growth, impaired muscle tone, persistence of juvenile hair, and failure of vaginal membrane rupture. Completness of the operation was further confirmed at autopsy by examination under a binocular dissecting microscope of the sella turcica. The atrophic condition of the pituitary target organs offered additional confirmatory evidence of completeness of hypophysectomy.

Thyroidectomy was accomplished surgically, the completeness of operation being judged on the basis of body growth. In completely thyroidectomized rats growth stasis was complete within 40 days at a maximum of 110 gm. Impaired muscle tone, persistence of juvenile hair and how metabolic rate gave additional confirmatory evidence. In some instances uptake of I^{131}, using a scintillation counter positioned over the neck of the living animal, was used in selecting animals for experimentation. The completeness of thyroid removal was checked routinely at the end of the experiment by the intraperitoneal administration of a tracer dose of I^{131} (1–2 μc.). Twenty-four hours later the rats were sacrificed and the entire ventral neck region was removed, placed in a vial, and the I^{131} uptake was counted directly in a scintillation counter. The difference between radioactivity per minute and that in a comparable piece of leg musculature gave the counts per minute taken up by any thyroid remnant. The uptake was then expressed as the percentage of the total quantity of radioactivity administered. All rats in which tissue from the neck region

incorporated more than 1% of that of the comparable region of the normal rat were considered incompletely thyroidectomized and rejected.

Adrenalectomy was performed through the lumbar approach. Adrenalectomized rats were given 1% NaCl solution in place of tap water to drink. Thyroidectomized-adrenalectomized rats were maintained on 1% NaCl solution or 0.1 mg. hydrocortisone acetate plus 0.1 mg. desoxycorticosterone acetate. At the end of the experimental procedures the rats were transferred to a high potassium, low sodium diet and the completeness of the operation was judged by the rapidity with which death ensued after this dietary substitution and the removal of maintenance therapy. This diet consisisted of alcohol extracted casein 24.0%, sucrose 63.6%, Crisco 8.0%, salts free of NaCl 3.3%, and K_2CO_3 1.1%. Crystalline vitamins per Kg. of diet were: 300 μg. d-biotin, 5 mg. 2-methyl-1,4 napthoquinone, 5 mg. thiamine HCl, 5 mg. pyridoxine HCl, 5.5 mg. pteroyglutamic acid, 10 mg. riboflavin, 10 mg. p-aminobenzoic acid, 20 mg. nicotinic acid, 50 mg. d-calcium pantothenate, 400 mg. inositol, 1.0 gm. choline chloride. K in the normal diet 0.616%, K in this diet approximately 1.3–1.4%. No data from animals which survived over seven days are included in the results. The presence of 1 mg. of adrenal cortical tissue permitted survival under this regimen for extended periods. As a further check on completeness, the adrenal site was examined for cortical remnants under a binocular dissecting microscope at autopsy.

Ovariectomy was performed through the usual lumbar approach. The completeness of the operation was determined by the failure of the vaginal membrane to rupture, and at autopsy by the atrophic condition of the uterus. In addition, a careful examination of the ovarian site was made.

Thyroidectomy-Adrenalectomy was performed in one operation. Completeness was judged by the same criteria given for the separate operations except in moribund animals were the terminal I^{131} uptake is not significant.

The hormonal preparations used and their standardization are as follows:

Thyrotrophic hormone was prepared by I. Gordon Fels from bovine pituitary glands according to the published method (16). Its potency was standardized in hypophysectomized rats by the histological response of the thyroids and by I^{131} uptake. By both of these criteria the preparation was effective at the minimal dose of 5–10 μg. It was free of follicle stimulating hormone, interstitial cell stimulating hormone, lactogenic hormone, adrenocorticotrphic hormone and growth hormone at the doses employed in this study. The hormone was administered by the intraperitoneal route in aqueous solution at pH 8.5.

The *adrenocorticotrophic preparations* used were: α-corticotropin (17) and fraction E (18) supplied by C. H. Li, α-pool corticotropin (19) supplied by Paul Bell of Lederle Laboratories, corticotropin B (20) supplied by Norman Brink of Merck and Company, Inc., and oxycellulose eluate (21)

prepared by Stanley Ellis. Preparations were suspended in 5% beeswax in peanut oil and administered subcutaneously in 0.1 cc.

The *melanocyte-stimulating hormone* was supplied by Irving I. Geschwind (22). It contained 1×10^6 units per gm. as determined by the method of Shizume, Lerner and Fitzpatrick, (23). It was contaminated with less than 0.1% of anterior pituitary hormones as determined in immature hypophysectomized rats. The hormone was suspended in 5% beeswax in peanut oil and administered subcutaneously in 0.1 cc.

Lactogenic hormone (prolactin) was prepared from sheep pituitary glands by R. David Cole (24). The preparation contained 35 I.U. per mg. The hormone was administered intraperitoneally in an aqueous solution at pH 8.5.

Stable suspensions of Merck *cortisone acetate* and *hydrocortisone acetate* were diluted with 0.9% NaCl, and injected subcutaneously in a volume of 0.1 cc.

Sodium L-triiodothyronine, supplied by Smith, Kline and French Laboratories, and *sodium L-thyroxine* were dissolved in alkaline 0.9% NaCl solution and injected intraperitoneally.

NOTES AND COMMENTS

ALDOSTERONE IN THE ADRENAL VEIN BLOOD OF NEPHROTIC RATS

It has been shown that the urinary excretion of aldosterone is increased in the nephrotic syndrome (1). It has been assumed that this phenomenon reflects an increase in the actual secretion of hormone by the adrenal gland. With the introduction of methods for the consistent production of experimental nephrosis in rats and the collection of adrenal vein blood of rats this hypothesis could be tested experimentally. Preliminary results reported here suggest that, in experimental nephrosis, there is an increase in the secretion of aldosterone by the adrenal gland.

METHODS

Rats were rendered nephrotic by the administration of an aminonucleoside related to puromycin as described by Frenk *et al.* (2) Males (Long-Evans strain) weighing 75–80 gm. were injected daily, s.c., for seven or nine days with 1.75 mg. of the drug (6-dimethylaminopurine, 3-amino-d-ribose, Lederle Laboratories, American Cyanamid Co.) in 0.2 ml. of water.

The procedure for the collection of adrenal vein blood and the measurement of the adrenal hormones was similar to that used in a previous study (3). In the present study the cannulae were made of capillary tubing and lined with silicone. The blood from several rats was pooled for extraction and assay. The number of rats used in each pool varied, depending on whether the aldosterone was expected to be high or low, and is indicated in the table. A standard procedure was used for the extraction and purification of all blood samples. The volumes of solvents used were constant relative to the volume of blood being extracted.

Residues were chromatographed on paper in a system suggested by Bush (4) consisting of toluene-75% aqueous methanol and run at 37°C after overnight equilibration. Part of the extract was used for estimation of corticosterone by the soda-fluorescence reaction (4). In the table, the value estimated to be the closest to the real value is recorded, together with the presumed extreme range.

The rest of the extract was chromatographed separately and the appropriate zone was eluted for bio-assay. The method of assay was that of Simpson and Tait (5) using the urinary Na-24 and K-42 ratio of adrenalectomized rats as an index of activity. Eight rats were generally used for assay at each dose level of standard or unknown, and three dose levels of the standard, desoxycorticosterone-acetate (DCA) were used on each day of assay. Rats of the Long-Evans strain were used in most instances. They were three and a half to four weeks old at operation.

RESULTS

With the amount of drug used in these experiments, edema was generally clearly visible after eight daily injections. On the ninth day, the treated animals weighed approximately the same as their untreated controls, i.e. about 100 gm., however, a large part of their body weight was composed of water in the form of edema and/or ascites. Histological sections have been prepared from the kidneys of some of these nephrotic rats. The most striking feature of these kidneys is the large amount of protein precipitate in the lumen of the tubules. Also there is a suggestion that there may be minor alterations in the basement membrane of the glomeruli. Examination of the sections is continuing.

Biochemical determinations were made on the blood and urine of nephrotic and normal rats, and the values obtained were similar to those reported by Frenk *et al.* (2). There was a marked increase in the plasma N.P.N. and cholesterol values, an increase in the plasma potassium and a decrease in the serum albumin in the nephrotic rats as compared with untreated control animals. The nephrotic rats also had marked albuminuria.

In order to overcome any seasonal or other differences which may have occurred throughout this study, blood from normal untreated animals was generally collected and assayed at the same time as the nephrotic rats. The results are expressed in terms of

μg/adrenal/kg rat weight/hr. for corticosterone, and as micrograms of DCA equivalents/adrenal/kg./hr. for aldosterone (Table 1).

Four groups of untreated animals have been studied. The aldosterone values ranged from 14 to 54.3 μg. DCA equivalents and the corticosterone values were between 132 and 266 μg.

Seven groups of nephrotic rats have been studied. In 2 experiments, the animals received 7 daily injections of the drug and the adrenal vein blood was collected on the 8th day. In these animals there was no obvious edema or ascites, although small (about 1 ml.) amounts of fluid were found in the abdominal cavity at operation. In 4 experiments the animals received 9 daily injections and the adrenal vein blood collections were made

TABLE 1. ALDOSTERONE AND CORTICOSTERONE IN THE ADRENAL VEIN BLOOD OF UNTREATED AND NEPHROTIC RATS

Treatment	Rats for adrenal effluent pool			Aldosterone/adr./kg./hr.		Corticosterone/adrenal/kg./hr.	
	No. of rats	Mean wt. (gm.)	Mean blood vol/adr./kg./hr. (ml.)	No. of rats/assay	Estimate* (μg DOCA equiv.)	Visual estimate (μg.)	Probable range (μg.)
Untreated Gp. I	6	105	18.3	8	14.0	266	200–336
Nephrotic—9 injections, studied 10th day	6	105	19.6	8	65.1	284	214–356
Untreated Gp. II	6	101	12.7	8	43.6	132	99–165
Nephrotic—9 injections, studied 10th day	7	108	16.5	8	91.3	500	374–625
Nephrotic—7 injections, studied 8th day	3	81	15.1	8	125.2	206	164–243
Untreated Gp. III	3	96	21.2	6	54.3	222	160–278
Nephrotic—7 injections, studied 8th day	3	69	17.4	6	167.9	338	290–386
Nephrotic—9 injections, studied 10th day	1	86	17.8	7	132.3	310	232–388
Nephrotic—9 injections, studied 11th day	2	81	16.5	6	465.3	495	330–660
Untreated Gp. IV	2	90	10.2	8	42.4	236	157–312
Nephrotic—9 injections, studied 10th day	2	98	15.6	8	170.0	238	204–272

* Calculated according to Irwin (6).

on the 10th day. In these animals the amount of ascitic fluid found in the abdominal cavity was generally between 5 and 15 ml. In another experiment the animals were given 9 daily injections and were studied on the 11th day. In these animals the volumes of ascitic fluid were slightly smaller than in the animals studied on the 10th day, but there appeared to be much more subcutaneous edema. The aldosterone values in these nephrotic rats ranged between 65.1 and 465.3 μg. of DCA equivalents. The corticosterone values were between 206 and 500 μg. In every instance the aldosterone levels found in the nephrotic rats were higher than those obtained in their respective control group, and the values in all seven groups were higher than the highest value obtained in any control group. In the experiment in which the animals were studied on the 11th day, the aldosterone secretion was almost 10 times as high as it was in the control group studied at the same time. In two of these experiments, the corticosterone levels were higher than those observed in the control animals.

The aldosterone values in the first group of untreated and nephrotic animals was somewhat low when compared with similar groups studied later, suggesting that there may be seasonal variation in the secretion of aldosterone. However, the difference between the untreated and nephrotic animals was still present. Comparison of estimates of untreated and nephrotic rats, with the exception of the very high value obtained in the nephrotic animals studied on the 11th day, gave a t value which was significant at the 1% level.

COMMENT

While it cannot be stated that the values obtained in this study represent the adrenal hormone secretion of normal and nephrotic rats under resting conditions, they do lend added support to the view that there is an increase in the secretion of aldosterone by the adrenal gland in this condition. It is noted that after 7 injections there was an increase in the secretion of aldosterone although there were no visible signs of ascites or edema. This suggests that in the experimental disease the increase in aldosterone secretion precedes the appearance of edema and ascites.

SUMMARY

Rats can be rendered nephrotic by the administration of an amino-nucleoside of puromycin and the hormone content of the adrenal venous blood determined.

In seven different experiments the aldosterone content of the adrenal venous effluent has been found to be higher than in normal rats studied at the same time. The values found in all the nephrotic animals were higher than those found in any of the control animals of the same weight range studied throughout the course of these experiments.

The results also suggest that in experimental nephrosis the increase in aldosterone secretion precedes the appearance of edema and/or ascites.

In two of seven experiments the corticosterone secretion was found to be somewhat higher than the highest value observed in untreated rats.

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SUPPRESSION OF CALCIUM-MOBILIZING ACTIVITY OF PARATHYROID EXTRACTS BY INORGANIC PHOSPHATE[1]

In the course of studies on the purification of parathyroid extracts there were occasions when it would have been convenient to assay the calcium-mobilizing activity of certain fractions in the presence of phosphate buffer. It was therefore important to determine whether or not inorganic phosphate would interfere with the calcium-mobilizing activity of the extract in rats. A preliminary report of this work has been published in the form of an abstract (1).

MATERIALS AND METHODS

The calcium-mobilizing activity was estimated by the serum calcium response of parathyroidectomized rats at the six hour post-operative period according to the biological assay method described by Munson *et al.* (2, 3). The standard parathyroid hormone preparation (P22) used was a crude extract prepared by heating ground, frozen bovine parathyroid tissue[2] with 0.1 N hydrochloric acid for 15 minutes at the temperature of a boiling water bath. When assayed against commercial U.S.P. extract[3] by the method just cited it was estimated to contain 35.8 ± 8.1 U.S.P. units/ml. (weighted mean of two assays). Both the commercial extract and P22 contained 0.2% phenol.

Phosphate was added as a solution of NaH_2PO_4, adjusted to pH 3.0 with hydrochloric acid and containing 8 mg. P/ml. Control solutions were prepared by dilution of the extract with acid saline (0.9% NaCl in 0.01 N HCl). Dialysis, when employed, was with cellulose, dialyzer tubing ($1\frac{3}{4}$ inches flat width) against 130 volumes of 0.001 N HCl, for 2 hours at 4° C, repeated once.

RESULTS

Experiments in which phosphate solution and parathyroid extract were mixed to give final concentrations ranging from 0 to 4.1 mg. P/ml., and 13 and 20 units/ml., respectively, are summarized in Table 1. A significant suppression of the calcium-mobilizing response was obtained in the presence of 4 mg. P/ml. at the six hour post-operative period.

In an attempt to elucidate the mechanism of this suppression effect three points were investigated: 1) the injection of extract and phosphate separately but simultaneously at different subcutaneous sites in the rat; 2) the levels of serum inorganic phosphate during this effect; and 3) dialysis of the extract-phosphate mixture after mixing and before assay. The injection of the extract and phosphate separately but simultaneously failed to show any suppression effect while the extract-phosphate mixture exhibited the usual effect (Fig. 1). Determination of serum inorganic phosphate levels demonstrated no significant differences between the three groups receiving extract (Fig. 2). Dialysis of the extract-phosphate mixture resulted in a response which was not significantly different from that of dialyzed extract alone or of the dialyzed extract and phosphate injected separately (Fig. 3). On the other hand a mixture of phosphate and previously dialyzed extract showed the usual lowered response. Analysis of the dialyzed extract-phosphate mixture and the dialyzed extract for inorganic phosphate revealed concentrations of 0.37 and 0.04 mg. P/ml. respectively showing that the dialysis had removed more than 90% of the added phosphate from the extract-phosphate mixture.

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[1] This work was supported in part by a grant from the American Cancer Society and by a research grant (D-203) from the National Institute of Dental Research of the National Institutes of Health, Public Health Service.

[2] Generously supplied by Eli Lilly and Company, Indianapolis, Indiana.

[3] Lilly Parathyroid Extract.

TABLE 1. SUPPRESSION BY PHOSPHATE OF SERUM CALCIUM RESPONSE TO PARATHYROID EXTRACT

Post-operative period, hours	PTE, units/ml.	Phosphate, mg. P/ml.	No. of rats	Serum calcium response, mg./100 ml. ±S.E.	
6	13	0	6	8.76±0.58	*
	13	4.1	6	6.29±0.30	
	20	0	3	8.89±0.46	**
	20	4.0	3	7.33±0.19	
	20	1.0	2	7.88±1.39	
	20	0.25	3	8.79±0.15	
3	20	0	5	8.68±0.77	**
	20	4.0	5	6.62±0.28	
	0	4.0	5	5.22±0.10	
12	20	0	5	7.85±0.42	**
	20	4.0	5	6.48±0.32	

Six hours is the usual post-operative period used in the bioassay method (2, 3). The suppressive effect is noted also at the 3- and 12-hour post-operative periods. The dose of parathyroid extract (PTE) was 1.0 ml./100 gm. body weight.

* $P < .01$.

** $P < .05$.

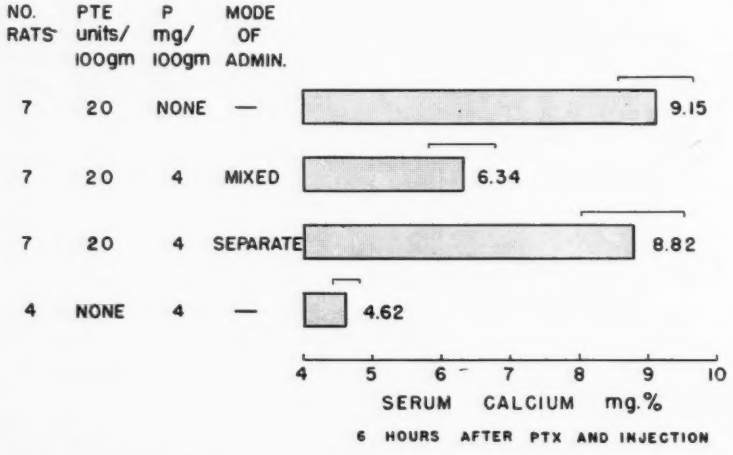

FIG. 1. Suppression of calcium-mobilizing activity by phosphate. The dose of parathyroid extract (PTE) was 1.0 ml./100 gm. body weight in each case. The parathyroid extract plus phosphate (P) were injected either mixed in a single injection or separately at different subcutaneous sites. Analysis of variance failed to show a significant difference between the first and third groups but revealed a difference between the second group and the combined first and third groups which was significant ($P. < 0.01$). The thin lines represent standard errors of the mean. PTX, parathyroidectomy.

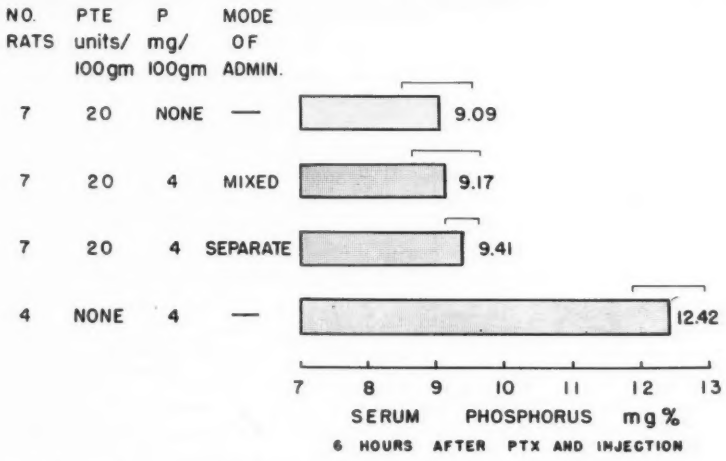
NO. RATS
PTE units/ 100gm
P mg/ 100gm
MODE OF ADMIN.
7 20 NONE — 9.09
7 20 4 MIXED 9.17
7 20 4 SEPARATE 9.41
4 NONE 4 — 12.42
7 8 9 10 11 12 13
SERUM PHOSPHORUS mg %
6 HOURS AFTER PTX AND INJECTION

FIG. 2. Serum inorganic phosphorus levels during suppression of the calcium-mobilizing activity by phosphate. The dose of parathyroid extract (PTE) was 1.0 ml./100 gm. body weight in each case. The parathyroid extract plus phosphate (P) were injected either mixed in a single injection or separately at different subcutaneous sites. Analysis of variance failed to show any significant difference between the first three groups. The thin lines represent standard errors of the mean. PTX, parathyroidectomy.

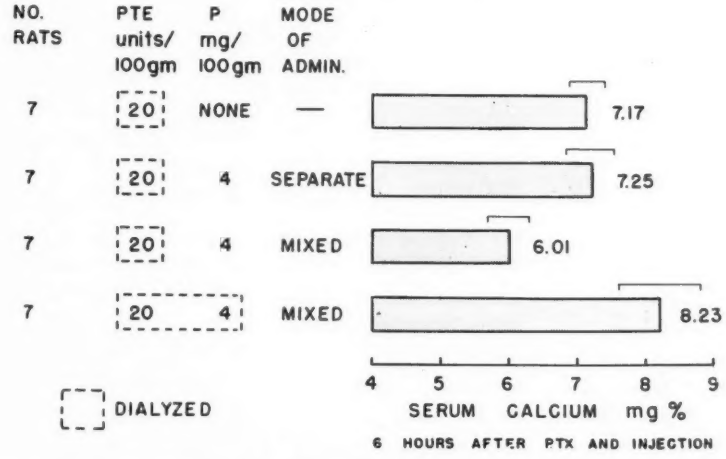
NO. RATS
PTE units/ 100gm
P mg/ 100gm
MODE OF ADMIN.
7 20 NONE — 7.17
7 20 4 SEPARATE 7.25
7 20 4 MIXED 6.01
7 20 4 MIXED 8.23
DIALYZED
4 5 6 7 8 9
SERUM CALCIUM mg %
6 HOURS AFTER PTX AND INJECTION

FIG. 3. Removal by dialysis of the effect of phosphate on the calcium-mobilizing activity. The dose of parathyroid extract (PTE) was 1.0 ml./100 gm. body weight in each case. The dialyzed parathyroid extract plus phosphate (P) were injected either mixed in a single injection or separately at different subcutaneous sites. Analysis of variance failed to show a difference between the first, second and fourth grpups but did reveal a difference between the third group and other three groups which was significant ($P<0.01$). The thin lines represent standard errors of the mean. PTX, parathyroidectomy.

DISCUSSION

It appears that caution has to to be exercised in the calcium-mobilizing assay of parathyroid extracts containing phosphate, for under certain conditions it is not possible to obtain the usual response. From the data presented it would seem that dialysis would be effective in overcoming this difficulty. However, because Kenny *et al.* (4) have shown that the calcium-mobilizing activity of crude parathyroid extracts is dialyzable to a considerable extent, dialysis would not be advisable.

In considering the mechanism of this suppression effect it was thought that perhaps the injection of phosphate had raised the serum inorganic phosphate level which by an inverse relationship, based on the idea of a constant calcium phosphate ion product, suppressed the rise in serum calcium. If this were so, injection of the same amount of phosphate at a subcutaneous site different from that of the extract alone should have the same effect. Besides, determination of the serum inorganic phosphate level should show a difference between the groups receiving and those not receiving phosphate As both these hypotheses proved to be unfounded a mechanism involving the serum phosphate level is unlikely. There exists the possibility that the reduction in calcium-mobilizing activity caused by phosphate is simply due to an effect on the rate of absorption of the parathyroid extract from the subcutaneous site, so that the maximum serum calcium response occurs either sooner or later than the six hour post-operative period used in the experiments described. An experiment in which the suppression effect of phosphate was noted at both the 3 hour and 12 hour post-operative periods, (Table 1) tended to rule out this possibility. Consequently, there appears to be a direct chemical interaction between phosphate and calcium-mobilizing hormone which suppresses its activity. Such interaction is reversed by dialysis.

SUMMARY

The influence of mixing phosphate with a crude parathyroid extract on the calcium-mobilizing activity of the latter in a rat bioassay method was studied.

In the presence of phosphate at a concentration of 4 mg. P/ml. the calcium-mobilizing activity of a crude parathyroid extract (20 units/ml.) was significantly reduced.

Injections of the same quantities of phosphate and extract simultaneously but separately at different subcutaneous sites failed to reduce the calcium-mobilizing activity.

The reduction of the calcium-mobilizing activity of parathyroid extract by mixture with phosphate was reversible by dialysis.

It is suggested that a direct interaction between the phosphate and the calcium-mobilizing hormone is responsible for the reduction in the activity of the latter.

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1. KENNY, A. D. AND P. L. MUNSON: *J. Dent. Res.* **34**: 701. 1955.
2. MUNSON, P. L., A. D. KENNY AND O. A. ISERI: *Fed. Proc.* **12**: 249. 1953.
3. MUNSON, P. L.: *Ann. New York Acad. Sc.* **60**: 776. 1955.
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THE ENDOCRINE SOCIETY

1957 Annual Meeting

The Thirty-ninth Annual Meeting of The Endocrine Society will be held in the Hotel New Yorker, New York, New York, Thursday, Friday, and Saturday, May 30, 31, June 1, 1957.

The Committee on Local Arrangements is Dr. Rulon W. Rawson as Chairman with Drs. Earl T. Engle, Joseph W. Jailer, Warren O. Nelson, and Martin Sonenberg as members of the Committee.

All Scientific Sessions will be held in the Hotel New Yorker. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 31, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the New Yorker, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Eleanor Venning, Royal Victoria Hospital, Montreal, Quebec, Canada, not later than February 1, 1957. *It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting*—in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.

Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter perfect, since there will be no opportunity for proof reading by authors.

Joint Sessions

The Endocrine Society will hold a joint session with the American Goiter Association on Thursday morning, May 30, 1957, and a joint session with the American Diabetes Association Saturday morning, June 1, 1957. Both of these joint sessions will be held in the meeting room of the Endocrine Society in the hotel New Yorker.

1957 LAURENTIAN HORMONE CONFERENCE

The 1957 Laurentian Hormone Conference of the AAAS will be held at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period September 1–6, and investigators interested in attending this conference should make applications to the Committee on Arrangements of The Laurentian Hormone Conference, 222 Maple Avenue, Shrewsbury, Massachusetts, at an early date and in any event no later than May 10, 1957. A conference rate of $12.00 per person per day is extended to all invited participants. Attendance is limited to invitations issued by the Committee on Arrangements. The following program has been arranged.

LAURENTIAN HORMONE CONFERENCE PROGRAM FOR 1957

I. *Hormone Structure and Function*

The chemistry and biological activities of 16-hydroxylated steroids.
Seymour Bernstein, Lederle Laboratories.
Sunday evening, September 1

Structure and hormonal activity of some new steroids.
Victor Drill and Byron Riegel, D. G. Searle & Co.
Monday morning, September 2

Relation of molecular structure of phenanthrene derivatives to growth.
Charles Huggins and Elwood V. Jensen, University of Chicago.
Monday morning, September 2

Studies on the mechanism of action of estrogen.
Gerald C. Mueller, University of Wisconsin.
Monday evening, September 2

II. *Hormones in Growth and Development*

Hormones and protein metabolism.
J. A. Leathem, Rutgers University.
Tuesday morning, September 3

Effects of hormones on growth in fattening and meat production potential of livestock (of domestic animals).
F. X. Gassner, E. C. Reifenstein, Jr., John W. Algeo and W. E. Mattox, Colorado A. & M.
Tuesday morning, September 3

The hormonal control of mammary growth and lactation.
William Lyons, University of Southern California.
Tuesday evening, September 3

III. *Aspects of Reproduction*

Cytological tests of chromosomal sex in relation to sexual abnormalities in man.
M. L. Barr and Melvin Grumbach, University of Western Ontario and Columbia University.
Wednesday morning, September 4

Clinical aspects of sexual abnormalities in man.
Robert Greenblatt, University of Georgia.
Wednesday morning, September 4

IV. *Hormones and Metabolism*

Effects of hormones on human serum lipoproteins.
Howard Eder, Albert Einstein College of Medicine.
Thursday morning, September 5

Effect of hormones on connective tissue metabolism.
Albert Dorfman and Sara Schiller, University of Chicago.
Thursday morning, September 5

Hormonal influences on connective tissue changes in aging.
Harry Sobel and Jessie Marmorston, Cedars of Lebanon Hospital.
Thursday evening, September 5

V. *Neurohumors*

Distribution and metabolism of adrenergic neurohumors in axones and tissues.
U. S. Von Euler, Stockholm, Sweden.
Friday morning, September 6

The excretion of epinephrine and norepinephrine under stress.
Fred Elmadjian, Justin M. Hope, Edwin T. Lamson, Worcester Foundation for Experimental Biology.
Friday morning, September 6

THE RESPONSIBILITIES OF THE MEDICAL PROFESSION IN THE USE OF X-RAYS AND OTHER IONIZING RADIATION

Statement by the United Nations Scientific Committee on the effects of Atomic Radiation

1. The United Nations General Assembly, being aware of the problems in public health that are created by the development of atomic energy, established a Scientific Committee on the Effects of Atomic Radiation. This Committee has considered that one of its most urgent tasks was to collect as much information as possible on the amount of radiation to which man is exposed today, and on the effects of this radiation. Since it has become evident that radiation due to diagnostic radiology and to radio-therapy constitutes a substantial proportion of the total radiation received by the human race, the Committee considers it desirable to draw attention to information that has been obtained on this subject.

2. Modern medicine has contributed to the control of many diseases and has substantially prolonged the span of human life. These results have depended in part on the use of radiation in the detection, diagnosis and treatment of disease. There are, however, few examples of scientific progress that are not attended by some disadvantages, however slight. It is desirable therefore to review objectively the possible present or future consequences of increased irradiation of populations which result from these medical applications of radiation.

3. It is now accepted that the irradiation of human beings, and particularly of their germinal tissues, has certain undesirable effects. While many of the somatic effects of radiation may be reversible, germinal irradiation normally has an irreversible and therefore cumulative effect. Any irradiation of the germinal tissues, however slight, thus involves genetic damage which may be small but is nevertheless real. For somatic effects there may however be thresholds for any irreversible effects, although if so these thresholds may well be low.

4. The information so far available indicates that the human race is subjected to natural radiation,[1] as well as to artificial radiation due to its medical applications, to atomic industry and its effluents and to the radioactive fall-out from nuclear explosions. The Committee is aware of the potential hazards that such radiation involves, and it is collecting and examining information on these subjects.

5. The amount of radiation received by the population for medical purposes is now, in certain countries, the main source of artificial radiation and is probably about equal to that from all natural sources. Moreover, since it is given on medical advice, the medical profession exercises responsibility in its use.

6. The Committee appreciates fully the importance and value of the correct medical use of radiation, both in the diagnosis of a large number of conditions, in the treatment of many such diseases as cancer, in the early mass detection of conditions such as pulmonary tuberculosis, and in the extension of medical knowledge.

7. Moreover, it appreciates fully the contribution of the radiological profession,

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[1] The radiation due to natural sources has been estimated to cause between 70 and 170 millirem of irradiation to the gonads per annum in most parts of certain countries in which it has been studied, although higher values are found locally in some areas. See the reports "The hazards to man of nuclear and allied radiations" published by the United Kingdom Medical Research Council in June 1956, in which also the millirem is defined; and from information submitted to the Committee.

through the International Commission on Radiological Protection[2] in recommending maximum permissible levels of irradiation. As regards those whose occupation exposes them to radiation, the establishment of these levels depends on the view that there are doses which, according to present knowledge, do not cause any appreciable body injury in the irradiated individual; and also on the consideration that the number of people concerned is sufficiently small for the genetic repercussions upon the population as a whole to be slight. Whenever exposure of the whole population is involved, however, it is considered prudent to limit the dose of radiation received by germinal tissue from all artificial sources to an amount of the order of that received from the natural background radiation.

8. It appears most important therefore that medical irradiations of any form should be restricted to those which are of value and importance, either in investigation or in treatment, so that the irradiation of the population may be minimized without any impairment of the efficient medical use of radiation.

9. The Committee is consequently anxious to receive information through appropriate governmental channels as to the methods and the extent by which such economy in the medical use of radiation can be achieved, both by avoiding examinations which are not clearly indicated and by decreasing the exposure to radiation during examinations, particularly if the gonads, or the foetus during pregnancy lie in the direct beam of radiation. It seeks, in particular, to obtain information as to the reduction in radiation of the population which might be achieved by improvements in instrument design by fuller training of personnel, by local shielding of the gonads, by choosing appropriately between radiography and fluoroscopy, and by better administrative arrangements to avoid any necessary repetition of identical examinations.

10. The Committee also seeks the co-operation of the medical profession to make possible an estimate of the total radiation received by the germinal tissue of the population before and during the child-bearing age. It considers it to be essential that standardized methods of measurement, of types at present available, should be widely used to obtain this information and it emphasizes the value of adequate records, maintained by those using radiation medically, by the dental profession, and by the responsible organizations in allowing such radiation exposure to be evaluated. The Committee is convinced that information of this type will make it possible to decrease the total medical irradiation of the population while preserving and increasing the true value of the medical uses of radiation.

GORDON RESEARCH CONFERENCES

The Gordon Research Conferences for 1957 will be held from 10 June to 30 August at Colby Junior College, New London, New Hampshire, New Hampton School, New Hampton, New Hampshire, and Kimball Union Academy, Meriden, New Hampshire.

The Conferences were established to stimulate research in universities, research foundations and industrial laboratories. This purpose is achieved by an informal type of meeting consisting of scheduled lectures and free discussion groups. Sufficient time is available to stimulate informal discussions among the members of a Conference. Meetings are held in the morning and in the evening, Monday through Friday, with the exception of Friday evening. The afternoons are available for recreation, reading or participation in discussion groups as the individual desires. This type of meeting is a valuable means of disseminating information and ideas which otherwise would not be realized through the normal channels of publication and scientific meetings. In addition,

[2] See the report of the International Commission on Radiological Protection (published in the British Journal of Radiology—Supp. 6, of December 1954—in the *Journal français d'electro-radiologie*—No. 10, of October 1955—etc. and revised in 1956).

scientists in related fields become acquainted and valuable associations are formed which result in collaboration and cooperative effort between different laboratories.

It is hoped that each Conference will extend the frontiers of science by fostering a free and informal exchange of ideas between persons actively interested in the subjects under discussion. The purpose of the program is not to review the known fields of chemistry, but primarily to bring experts up to date as to the latest developments, analyze the significance of these developments, and to provoke suggestions as to underlying theories and profitable methods of approach for making new progress. In order to protect individual rights and to promote discussion, it is an established rule of each Conference that all information presented is not to be used without specific authorization of the individual making the contribution, whether in formal presentation or in discussion. No publications are prepared as emanating from the Conferences.

The first meeting of each Conference is held Monday morning at 9:00 a.m., Eastern Daylight Saving Time. The morning sessions, through Friday, are scheduled from 9:00 a.m. to 12:00 noon. The second session of each day is held in the evening from 7:30 to 10:00 p.m., Monday through Thursday. There are no Friday evening meetings.

Accommodations are available for a limited number of women to attend each conference, also for wives who wish to accompany their husbands. All such requests should be made at the time of the request for attendance because these limited accommodations will be assigned in the order that specific requests are received. Children twelve years of age and older can be accommodated. Dogs or other animals will not be permitted in the dormitories.

Individuals interested in attending the Conference should request an application form from the office of the Director. Attendance at each Conference is limited to 100.

Requests for attendance at the Conferences, or for any additional information should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. From June 10 to August 30, 1957 mail should be addressed to Colby Junior College, New London, New Hampshire.

CHEMISTRY, PHYSIOLOGY AND STRUCTURE OF BONES AND TEETH

D. HAROLD COPP, Chairman, D. DZIEWIATKOWSKI, Vice-Chairman

15 July
ROBERT A. ROBINSON, Chairman. Fine Structure of Bones and Teeth.
ISIDORE GERSH. Fine Structure of Ground Substance.
WILLIAM F. NEUMAN, Chairman.
M. J. DALLEMAGNE. Nature of the Mineral of Bones and Teeth.
16 July
FRANKLIN C. MCLEAN, Chairman. Parathyroids and Regulation of Blood Calcium.
ROY V. TALMAGE. Calcium Regulation in the Rat Studies by Intraperitoneal Lavage.
GORDON S. STEWART. The Role of Parathyroids in Calcium Homeostasis.
D. HAROLD COPP. Regulation of Blood Calcium in the Dog.
WALLACE D. ARMSTRONG, Chairman.
BERTIL LINDQUIST. Studies on Calcium and Phosphorus Kinetics in Man.
17 July
WILLIAM P. NORRIS, Chairman. Radiostrontium Metabolism.
CYRIL L. COMAR. Comparison of the Biological Behaviour of Calcium and Strontium.
HAROLD C. HODGE, Chairman. General Topics.
Selected Short Papers.
17 July
HERMANN LISCO, Chairman.
JANET VAUGHAN. Radiation Effects on Bone.
18 July
ANN BUDY, Chairman. Effect of Hormones on the Skeleton.
MARSHALL R. URIST. The Transport of Calcium in the Blood of the Estrogenized Fowl.
C. WILLETT ASLING. Changes in Skeletal Tissues Induced by Pituitary Growth Hormone.
ISAAC SCHOUR. The Effects of Hormones on Teeth.
EDWARD C. REIFENSTEIN, JR., Chairman.
CHARLES E. DENT. Studies on Metabolic Bone Disease.
19 July
ALEXANDER B. GUTMAN, Chairman. Enzymes of Bone.
Subjects and Speakers to be Announced.